

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Title: METHOD FOR THE DEVELOPMENT OF AN HIV VACCINE

Appl. No.: 10/667,534
Applicant: Adan Rios
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Art Unit: 1648
Examiner: Parkin, Jeffrey S.
Docket No.: RIOS:004USC2
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January 23, 2008
Date


Travis M. Wohlers

APPEAL BRIEF

MAIL STOP APPEAL BRIEF - PATENTS

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Appellant submits this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Office Action dated June 15, 2007. Appellant filed a Notice of Appeal and Request for Pre-Appeal Brief Review on October 12, 2007. The Notice of Panel Decision from Pre-Appeal Brief Review was mailed November 23, 2007. Thus, the deadline for filing the Notice of Appeal was December 23, 2007. A request for a one-month extension of time is included, which brings the deadline for filing the Appeal Brief to January 23, 2008. The fees for filing the Appeal Brief and for the two-month extension of time are included. Should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/RIOS:004USC2.

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I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Photoimmune Biotechnology, Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 40-50 are currently pending and are rejected. Claims 1-39 have been canceled. The rejection of claims 40-50 is being appealed.

IV. STATUS OF AMENDMENTS

No amendments are pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER¹

Independent claim 48 is directed to a method of eliciting an immune response (Specification, p. 13, ln. 4-6) comprising: obtaining a viral particle comprising a reverse transcriptase that has been inactivated by binding said reverse transcriptase with one or more azido-labeled compounds and then irradiating said reverse transcriptase (Specification, p. 11, ln. 28-29; p. 12, ln. 8-9; p. 16, ln. 20-23); and administering the viral particle to a subject, wherein an immune response is elicited in the subject (Specification, p. 13, ln. 4-6).

Dependent claim 43 is directed to the method of claim 48, wherein the viral particle is an HIV particle (Specification, p. 11, ln. 7-10). Claim 44 depends from claim 43 and specifies that the HIV particle is HIV-1 (Specification, p. 12, ln. 2). Claim 45 depends from claim 44 and specifies that the HIV-1 is Group M or Group O (Specification, p. 12, ln. 2-3). Claim 46

¹ Parentheticals citing to support in the specification for the claim language are exemplary and not meant to indicate that the specific citations are the only support in the specification for the claim language.

depends from claim 45, and specifies that Group M is selected from the group consisting of clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, and clade I (Specification, p. 12, ln. 3-5). Claim 47 depends from claim 45, and specifies that the Group M particles are clade B particles (Specification, p. 12, ln. 5-6).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 40-50 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not reasonably described in the specification.

VII. ARGUMENT

A. *Claims 40-50*

The only issue on appeal is whether current claims 40-50 are supported by adequate written description in the specification as required by 35 U.S.C. § 112, first paragraph. The examiner alleges that the specification does not provide adequate written description of the recitation “a viral particle comprising a reverse transcriptase” in claim 48. Rather, the examiner contends that the claims should be limited to an HIV particle comprising an HIV reverse transcriptase (RT). The examiner’s position is based on a legally incorrect application of the written description requirement of 35 U.S.C. § 112, first paragraph.

The present specification teaches that a reverse transcriptase may be inactivated by binding the reverse transcriptase with one or more azido-labeled compounds and then irradiating it (*see e.g.*, p. 12, ln. 8-9). While HIV is used to exemplify the teachings in the specification, the specification specifically states that “the methodology of the present invention is *applicable to any retrovirus* which may be associated with any animal or human disease as a method for development of effective immunogens and preventative vaccines. *Thus, the present invention has a broader applicability than the exemplified HIV vaccine.*” (p. 16, ln. 20-23). The

examiner is improperly attempting to limit Appellant's claims to a preferred embodiment when the specification's disclosure explicitly states a broader application of the disclosed methods.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991). In addition to the express statement in the specification that "the present invention has a broader applicability than the exemplified HIV vaccine" (p. 16, ln. 20-23), one skilled in the art would have reasonably concluded that the inventor had possession of the currently claimed method for at least the following reasons.

As explained in the specification, HIV is a retrovirus and a unique aspect of retrovirus replication is the conversion of a single-stranded RNA from the virus genome into a double-stranded DNA molecule that must integrate into the genome of the host cell prior to the synthesis of viral proteins and nucleic acids (Specification, p. 3, ln. 4-12). Accordingly, all retroviruses possess a reverse transcriptase enzyme, which converts the RNA of their genetic material into DNA (Specification, p. 3, ln. 14-16). Furthermore, since all reverse transcriptases prime the synthesis of new DNA from tRNA, which is a molecule with abundant secondary structure strongly associated with the enzyme, it is generally accepted that the catalytic unit among reverse transcriptases is phylogenetically conserved (*see e.g.*, Flavell, Retroelements, reverse transcriptase and evolution, *Comp. Biochem. Physiol.* vol 110B,N01 pp3-15, 1995 (Evidence Appendix – Exhibit 1); Boeke, The unusual phylogenetic distribution of retrotransposons: A hypothesis, *Genome Res.* 2003 13:1975-1983 (Evidence Appendix – Exhibit 2); Nakamura *et al.*, Telomerase catalytic subunit homologs from fission yeast and human, *Science* vol.277, August 15 1997 (Evidence Appendix – Exhibit 3); Springer *et al.*, Phylogenetic relationships of reverse

transcriptase and RNase H sequences and aspects of genome structure in the gypsy group of retrotransposons, *Mol. Biol. Evol.* 10(6):1370-1379, 1993 (Evidence Appendix – Exhibit 4); Lingner *et al.*, Reverse transcriptase motifs in the catalytic subunit of telomerase, *Science* 276:561(1997) (Evidence Appendix – Exhibit 5); Valverde-Garduno *et al.*, Functional analysis of HIV-1 reverse transcriptase motif C: site directed-mutagenesis and metal cation interaction, *J. Mol. Evol.* 1998 Jul; 47(1):73-80 (Evidence Appendix – Exhibit 6); Seifarth *et al.*, Rapid identification of all known retroviral reverse transcriptase sequences with a novel versatile detection assay, *AIDS Research and Human Retroviruses*, vol. 16 Number 8, pp 721-729, 2000) (Evidence Appendix – Exhibit 7).

Since retroviruses cannot integrate into the genetic machinery of the host cell without reverse transcription, the inhibition of reverse transcriptase has as a universal consequence on the inability of any retrovirus to integrate within the genetic machinery of a suitable host cell. Thus, regardless of the type of retrovirus, the inactivation of reverse transcriptase as described in the present specification would be understood by a person of ordinary skill in the art to be applicable to any retrovirus. The importance of RT to retroviruses in general, is further evidenced by the number of known anti-retroviral compounds that interfere with RT activity (e.g., AZT, nevirapine, pyridinones, carboxanilides) (Specification, p. 3, ln. 23 to p. 4, ln. 8).

As described in the present specification, a reverse transcriptase may be inactivated by binding the reverse transcriptase with one or more azido-labeled compounds and then irradiating it (*see e.g.*, p. 12, ln. 8-9). Numerous compounds that bind to reverse transcriptases were known in the art (*see e.g.*, Specification, p. 3, ln. 23, to p. 4, ln. 11). Furthermore, in view of the phylogenetic conservation among reverse transcriptases (*see* Evidence Appendix – Exhibits 1-7) and the fact that all reverse transcriptases prime the synthesis of new DNA from a RNA

molecule with abundant secondary structure strongly associated with the enzyme, those of ordinary skill in the art would have known that a compound that binds to one RT would generally be expected to bind to other RTs as well. For example, many anti-retroviral compounds are nucleoside analogs, such as AZT, which inhibit the reverse transcriptase by competing with the naturally occurring deoxynucleotides needed to synthesize the viral DNA. All reverse transcriptases need deoxynucleotides to synthesize viral DNA; thus, a person of ordinary skill in the art would have understood that a nucleoside analog that competed with the naturally occurring deoxynucleotides to inhibit one RT would also be expected to bind to and inhibit other RTs, and that the inhibition of RT has the universal consequence of inhibiting the ability of retroviruses to integrate into the DNA of a host cell and replicate.

As mentioned above, numerous compounds known to bind reverse transcriptases were known in the art and are disclosed in the specification (Specification, p. 3, ln. 27, to p. 4, ln. 1; pg. 12, ln. 14-18; p. 21, ln. 21-26). The specification teaches that these compounds may be converted to azido photoaffinity labels and utilized for the inactivation of reverse transcriptase (Specification, p. 21, ln. 21-28). Thus, it would have been understood by a person of ordinary skill in the art at the time of filing that the inactivation of reverse transcriptase as described in the present specification would be generally applicable to viral particles comprising a reverse transcriptase.

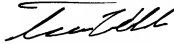
In view of the above, the present specification describes the claimed invention in sufficient detail that one of ordinary skill in the art can reasonably conclude that Appellant had possession of the claimed invention at the time of filing. Appellant, therefore, requests that the Board overturn this rejection.

B. *Claims 43-47*

Appellant separately argues dependent claims 43-47. The examiner stated that claims directed towards “a method of eliciting an immune response by administering a human immunodeficiency virus (HIV) particle comprising an HIV reverse transcriptase (RT) that has been inactivated” are supported by the specification (Action, p. 5). Dependent claim 43 is directed to the method of claim 48, wherein the viral particle is an HIV particle. Claim 44 depends from claim 43 and specifies that the HIV particle is HIV-1. Claim 45 depends from claim 44 and specifies that the HIV-1 is Group M or Group O. Claim 46 depends from claim 45, and specifies that Group M is selected from the group consisting of clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, and clade I. Claim 47 depends from claim 45, and specifies that the Group M particles are clade B particles.

In rejecting a claim under the written description requirement, the examiner has the initial burden of presenting evidence or reasons why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined in the claims. Here, the examiner has not only failed to establish a lack of written description for claims 43-47, he has expressly stated that the claims are supported by adequate written description. Accordingly, the rejection of claims 43-47 should be overturned.

Respectfully submitted,



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Date: January 23, 2008

VIII. APPENDIX A – APPEAL CLAIMS

40. The method of claim 48, wherein said azido-labeled compound is azido dipyridodiazepinona or *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide.
41. The method of claim 48, wherein said azido-labeled compound is *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide.
42. The method of claim 48, wherein the irradiation is with UV light.
43. The method of claim 48, wherein the viral particle is an HIV particle.
44. The method of claim 43, wherein said HIV particle is HIV-1.
45. The method of claim 44, wherein said HIV-1 is Group M or Group O.
46. The method of claim 45, wherein said Group M are selected from the group consisting of clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, and clade I.
47. The method of claim 45, wherein said Group M particles are clade B particles.
48. A method of eliciting an immune response comprising:
obtaining a viral particle comprising a reverse transcriptase that has been inactivated by binding said reverse transcriptase with one or more azido-labeled compounds and then irradiating said reverse transcriptase; and
administering the viral particle to a subject, wherein an immune response is elicited in the subject.
49. The method of claim 48, wherein the subject is human.
50. The method of claim 49, further defined as a method of vaccination.

IX. APPENDIX B - EVIDENCE APPENDIX

Exhibit 1: Flavell, Retroelements, reverse transcriptase and evolution, *Comp. Biochem. Physiol.* vol 110B,N01 pp3-15, 1995. First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 2: Boeke, The unusual phylogenetic distribution of retrotransposons: A hypothesis, *Genome Res.* 2003 13:1975-1983. First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 3: Nakamura *et al.*, Telomerase catalytic subunit homologs from fission yeast and human, *Science* vol.277, August 15 1997. First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 4: Springer *et al.*, Phylogenetic relationships of reverse transcriptase and Rnase H sequences and aspects of genome structure in the gypsy group of retrotransposons, *Mol. Biol. Evol.* 10(6):1370-1379, 1993. First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 5: Lingner *et al.*, Reverse transcriptase motifs in the catalytic subunit of telomerase, *Science* 276:561(1997). First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 6: Valverde-Garduno *et al.*, Functional analysis of HIV-1 reverse transcriptase motif C: site directed-mutagenesis and metal cation interaction, *J. Mol. Evol.* 1998 Jul; 47(1):73-80. First cited in the Information Disclosure Statement filed March 5, 2007.

Exhibit 7: Seifarth *et al.*, Rapid identification of all known retroviral reverse transcriptase sequences with a novel versatile detection assay, *AIDS Research and Human Retroviruses*, vol. 16 Number 8, pp 721-729, 2000). First cited in the Information Disclosure Statement filed March 5, 2007.

X. APPENDIX C - RELATED PROCEEDINGS

None

EXHIBIT 1



Pergamon

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INVITED REVIEW

Retroelements, reverse transcriptase and evolution

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Retroelements are genetic elements that can exist as DNA or RNA or DNA/RNA duplexes. Although retroviruses are the best known retroelements, there are many other types, including close relatives of retroviruses like LTR retrotransposons, more distant relatives like non-LTR retrotransposons, caulimoviruses and hepadnaviruses and elements with virtually no similarity, like retrons. Virtually all retroelements are 'selfish DNAs' with no involvement with the normal development or maintenance of their host cells, the only known exception being telomerases/telomerases which maintain the ends of chromosomes. Virtually all retroelements use tRNA, or RNA with strong secondary structure, to initiate their reverse transcription. The coincidence between the use of tRNA, a molecule central to the conversion of RNA to protein, with reverse transcriptase, an enzyme which is crucial for the conversion of RNA to DNA is striking, because RNA probably preceded DNA and protein in evolution. It seems plausible that retroelements were present at the genesis of living systems.

Key words: Retrovirus; Reverse transcriptase; Retrotransposon; Retron; Retroelement; Evolution; Telomerase; Caulimovirus; Hepadnavirus; Pararetrovirus.

Comp. Biochem. Physiol. 110B, 3-15, 1995.

Introduction

This review is intended to give the reader an overview of the many apparently diverse manifestations of genetic elements containing reverse transcriptase. What emerges from a closer look at these elements is the striking similarity between many of them, suggesting that many, and perhaps all these elements share a common evolutionary origin.

The Origin of Reverse Transcriptase

RNA has an intrinsic catalytic ability to make and break its own phosphodiester backbone. We, therefore, believe that RNA was probably the first self-replicating entity and evolution first worked on it before DNA and protein were brought into the picture (Cech and Bass, 1986; Darnell and Doolittle, 1986).

Although RNA was probably the first genetic material, it is poorly suited to that role because it is chemically labile. DNA is much more inert and better suited to carrying genetic information between generations. RNA can be converted to DNA by reverse transcriptase, an enzyme which is related in sequence to RNA replicases

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Table 1. Types of retroelement

Telomeres/telomerase
Group II introns
Retrons
Fungal mitochondrial plasmids
Non-LTR retrotransposons
LTR retrotransposons
Retroviruses
Caulimoviruses and hepadnaviruses

which exist in RNA viruses (Xiong and Eickbush, 1990). We believe that reverse transcriptase evolved from an RNA replicase and this event was central to the development of DNA as the main form of genetic material in living organisms.

Once reverse transcriptase had accomplished this feat, it largely bowed out of the main story of evolution, leaving DNA as the genetic store and RNA as either the message for production of proteins (messenger RNA) or part of the machinery of RNA splicing, polyadenylation, etc, catalysed by ribonucleoprotein complexes, and translation, catalysed by transfer RNAs and ribosomal RNAs. But it did not disappear entirely and has been found still in a wide variety of guises. These 'retroelements' are listed in Table 1 and I will first review them in ascending order of sophistication, concentrating mainly on what we know of their replication cycles, before discussing the evolutionary implications of these data.

Telomeres and Telomerases

Telomeres are the extreme ends of linear chromosomes. Linear chromosomes are ubiquitous (as far as we know) in nuclear eukaryotes genomes and they face the same dilemma found by all linear DNAs, namely, how to resist agents which lead to shortening, such as attack by DNA exonucleases or the inherent inability of DNA polymerase to copy its template to its extreme 5' end. The basic solution to the problem for the majority of eukaryotes is to locate a simple sequence at the telomere which counteracts the reduction in size by replicating extra copies of itself (Blackburn, 1992; Schippen, 1993). The exact sequences of the repeats within telomeres are species-specific, but a typical example is that of *Tetrahymena thermophila*, in which the sequence GGGGGTTT is repeated many times.

The template for these extra copies is an RNA molecule which is bound tightly to a specialised type of reverse transcriptase called telomerase. In this case, the template RNA carries a homologous sequence (5'AACCCCAA3') which is used to generate a new DNA strand (Fig. 1).

There is still very little known about the telomerase enzyme which carries out this function, because it is difficult to work with. An exciting possibility is that the RNA template itself is directly involved in the catalytic process, making it another RNA enzyme (ribozyme). However, this hypothesis remains untested and no ribozyme to date has been shown to catalyse reactions on DNA. Perhaps RNA never learned this feat and DNA has only ever been 'genetically manipulated' by proteins.

Group II Introns

Two revolutionarily ancient types of intron survive in the organelles or plasmids of lower eukaryotes. These group I and II introns have the ability to splice themselves out of their precursor mRNAs, without the help of proteins (Cech and Bass, 1986). The two groups are classified by their characteristic sequence motifs which themselves define group-specific conserved secondary structures in the intron RNA. Several of these introns encode 'maturase' genes which aid the splicing process in the cell, although the polypeptides encoded by these genes are not essential for self-splicing in the test tube (Carignani *et al.*, 1983). A variety of polypeptides are used, these are derivatives of enzymes concerned with RNA metabolism (Lambowitz and Perlman, 1990).

Polypeptides resembling aminoacyl tRNA synthetases are common and reverse transcriptase-like proteins are also found. These reverse transcriptase-like proteins have not yet been shown to have enzymic activity but another strange property of group II introns suggests that this may be the case. Some group II introns occasionally transpose to new chromosomal locations (Mueller *et al.*, 1993; Sellem *et al.*, 1993). These new locations are sometimes non-homologous to pre-existing insertion sites for the introns, suggesting that this is true transposition and not a phenomenon related to homologous recombination.

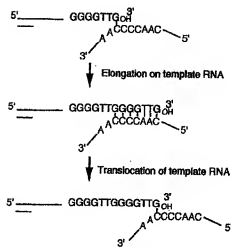


Fig. 1. Mechanism for elongation of telomere repeats by telomerase. DNA is shown in black and RNA in red.

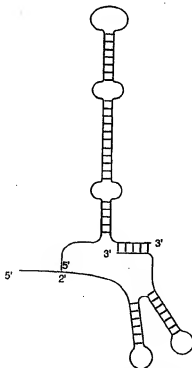


Fig. 2. msDNA. Base pairs involved in secondary structure are shown. DNA is shown in black and RNA in red.

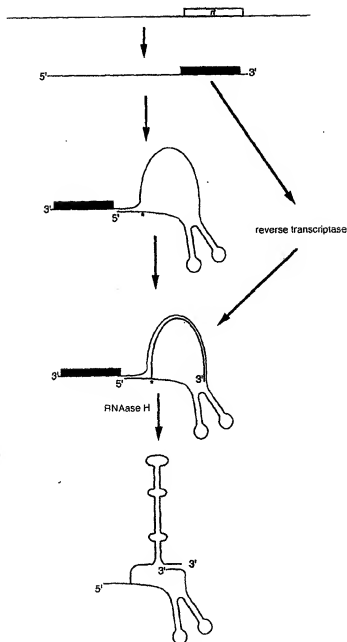


Fig. 3. Biosynthesis of msDNA from its retron template. DNA is shown in black and RNA in red. Initiation of reverse transcription is from the 2' OH group of a G base (indicated by *).

While other models are possible, it is feasible that the transposition occurs in the following way. First, an RNA copy of the intron becomes inserted into another RNA by a reversal of the normal splicing mechanism (such reverse-splicing has already been demonstrated in group II introns; Augustin *et al.*, 1990). Then the novel intron-containing RNA is reverse transcribed into DNA which then either recombines into the chromosomal DNA directly or by gene conversion.

Retrons

The bizarre RNA-DNA chimaera shown in Fig. 2 is found in many bacteria (Inouye and Inouye, 1993). This small extrachromosomal molecule, called msDNA, is synthesized in bacterial strains containing a genetic element called a retron (Fig. 3). A retron minimally consists of the DNA template for msDNA synthesis, plus a reverse transcriptase gene. A single promoter transcribes the entire genetic element,

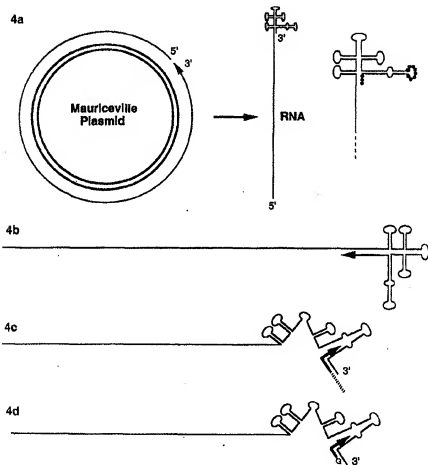


Fig. 4. The Mauriceville mitochondrial plasmid of *Neurospora*. (a) Secondary structure of the RNA transcript. RNA is shown by thin lines and DNA by thick lines. Bases conserved with tRNAs are shown by small black circles. (b) *In vitro* initiation of reverse transcription by elongation primed from the 3' end of the RNA. (c) *In vitro* initiation of reverse transcription by a non-specific DNA oligonucleotide (shown by a striped line). (d) *In vitro* initiation of reverse transcription by elongation primed by G mononucleoside or mononucleotide.

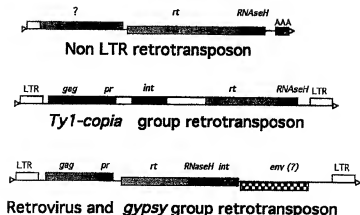


Fig. 5. Gene structural features of retrotransposons and retroviruses. See text for functions of genes.

producing a long transcript from which reverse transcriptase is synthesized. The transcript can adopt a secondary structure, because it contains several regions of self-complementarity. This folded RNA then acts as a template for reverse transcription by the retron-encoded enzyme. This synthesis seems to be primed from a 2' OH group (starred in Fig. 3), unlike all other known reverse transcriptases, RNA polymerases and DNA polymerases, which prime from 3' OH groups. The reverse transcription becomes stalled at one of the RNA hairpin loops. Degradation of most of the RNA part of the resulting heteroduplex leads to the mature msDNA molecule.

What is the point of this strange phenomenon? Only a proportion of the members of a retron-containing bacterial species actually contain retrons. We are therefore confident that this genetic element is a kind of 'selfish DNA' (Doolittle and Sapienza, 1980; Orgel and Crick, 1980; Sapienza and Doolittle, 1981) which is non-essential for the host and whose prime function is its own propagation. It seems unlikely that msDNA is an intermediate in an extrachromosomal replication cycle of retrons, because large parts of the complete retron are missing from it (including the reverse transcriptase gene) but it may be the abortive descendant of such an intermediate. We shall see below that most retroelements use reverse transcription to replicate themselves via extrachromosomal intermediates.

Fungal Mitochondrial Plasmids

Certain fungi (notably some *Neurospora* species) sometimes contain small circular double stranded plasmids in their mitochondria. The best characterized of these (the Mauriceville and Varkud plasmids) have been shown to contain reverse transcriptase genes and to be replicated by a transcription-reverse transcription cycle (Fig. 4). Transcription of the plasmid yields an RNA of exactly the same size as the plasmid, with an intriguing secondary structure at the 3' end which is reminiscent of tRNAs and the 3' ends of RNA viruses of plants and bacteria; indeed, several bases conserved among tRNAs (shown by small black circles in the figure) are found in the plasmid RNA (Fig. 4a). *In vitro* studies using the purified mitochondrial enzyme has shown that reverse transcription can be initiated in three different ways (Wang and Lambowitz, 1993). The first involves elongation from the 3' end of the RNA, perhaps via the secondary structure shown in Fig. 4b. The second way is by elongation of a short, non-specific DNA oligonucleotide which is bound to the reverse transcriptase (Fig. 4c). This time DNA synthesis begins at the penultimate base of the RNA. The final way (Fig. 4d) is identical to the second, except that no exogenous DNA primer is required, just a G base, dGMP, dGDP and dGTP all function in this *in vitro* reaction. It is unclear which, if any of these methods of reverse transcription initiation

predominates *in vivo*. At present, we do not know how the full DNA-RNA hybrid which is synthesized as the first step in the reverse transcription process, is eventually converted into the circular double stranded plasmid.

Non LTR Retrotransposons

Retrotransposons are transposable genetic elements which use reverse transcription during their movement to new chromosomal locations. Non-LTR retrotransposons are the simplest type of retrotransposon. They are found in the genomes of the majority of eukaryotes (including mammals, where they are called LINE, L1 or Kpn elements; Hutchinson *et al.*, 1989; Martin, 1991). They contain two discernible genes, one of which is a reverse transcriptase/RNase H gene (*rt RNaseH*; Fig. 5). A single transcript, initiating at the exact 5' boundary of the retroelement, encodes all the genetic information of the element (Mizrokhi *et al.*, 1988). The details of the transposition cycle are still unclear but a particle, comprised of proteins encoded by the other gene in the element (marked by ? in the figure) and containing the retrotransposon's RNA and reverse transcriptase, has been identified and is perhaps involved in the reverse transcription and integration process, just as the analogous proteinaceous particles are implicated in the transposition cycles of LTR retrotransposons and retroviruses (see below; Deragon *et al.*, 1990; Martin and Branciforte, 1993). The exact reverse transcription mechanism for non-LTR retrotransposons is still unclear, but is believed that the reverse transcript becomes inserted at random nicks in the chromosome (Finnegan, 1989).

LTR Retrotransposons and Retroviruses

These two types of retroelement are so similar that I will consider them together. The gene structures of retrotransposons and the DNA forms of retroviruses (called proviruses) are shown in Fig. 5. There are two main groups of LTR retrotransposons, the *Ty1-copia* group and *gypsy* group, named after prototype elements in yeast

and *Drosophila*, respectively (Bingham and Zachar, 1989). The former is structurally the more simple group. Both groups are found in fungi, plants and insects. There has been some debate about the existence of retrotransposons in vertebrates. Early claims were probably no more than defective retroviruses (Hodgson *et al.*, 1990) but recent comprehensive PCR-based searches have identified *Ty1-copia* group retrotransposons in fish, amphibia and reptiles (though not mammals and birds as yet; Flavell and Smith, 1992; Flavell *et al.*, 1994).

LTR retrotransposons are known to use intracellular ribonucleoprotein particles as intermediates in their transposition cycles (Shiba and Saigo, 1983; Garfinkel *et al.*, 1985). The protein structural components of the particles are encoded by the *gag* genes (see Fig. 5). The particles also contain virtually full length transcripts of the transposons and several enzymes, all of which are retrotransposon-encoded. A protease (encoded by the *pr* gene) is involved in cleaving the precursor polyprotein into the mature proteins, a reverse transcriptase first copies the transposon RNA to form an RNA-DNA duplex. For all LTR retrotransposons and retroviruses, initiation of the reverse transcription is from a 3' end of tRNA physically bound to the template and the enzyme (Bingham and Zachar, 1989; Varmus and Brown, 1989). The next step is the degradation of the RNA in the duplex by ribonuclease H to enable synthesis of the second DNA strand by the reverse transcriptase. Finally, an integrase (encoded by the *int* gene) catalyses the insertion of the double-stranded DNA copy into the chromosome.

Retroviruses use just the same genes to achieve the same result as LTR retrotransposons (Varmus and Brown, 1989). The only significant functional difference between the two is the retroviruses proven ability to leave the cell as a virus particle. Entry of virus particles into a new cell requires an envelope glycoprotein, encoded by the *env* gene (Fig. 5), which is embedded in the plasma membrane envelope acquired by the virus when it buds out through the cell membrane. In fact, *gypsy* group retrotransposons are believed by some to actually be retroviruses. They possess an extra

gene of unknown function in the same location as *env* and there is some evidence to suggest that the *gypsy* transposon of *Drosophila* can form an infectious particle (Kim *et al.*, 1994).

Hepadnaviruses and Caulimoviruses (Pararetroviruses)

Two other virus groups use reverse transcription in their life cycles, though neither normally integrate their DNAs into the host chromosomes. Hepadnaviruses (hepatitis B-like viruses) infect vertebrates. They contain a small circular DNA molecule which is partially single-stranded (Fig. 6; Tiollais *et al.*, 1981). This DNA encodes genes specifying a reverse transcriptase, capsid structural components and viral surface proteins. Upon infection, the virion DNA is filled in by the encapsidated reverse transcriptase to form a closed circular double stranded DNA. Transcription of this template yields an RNA which is translated into the virus-encoded proteins (Summers and Mason, 1982). Initiation of reverse transcription is primed by a protein bound to the virion RNA, unlike all other retroelements which use RNA primers (Gerlich and Robinson, 1980). Reverse transcription commences in the particle but remains incomplete, forming the partially single-stranded virion DNA.

Caulimoviruses are plant viruses which share with hepadnaviruses the properties of encapsidation of an incompletely reverse transcribed DNA (Bonneville *et al.*, 1988). In this case, the capsid nucleic acid is largely double-stranded with a few nicks (Fig. 6). The basic steps of transcription from an extrachromosomal closed circular DNA template into RNA which is translated into reverse transcriptase and virus particle components are shared with hepadnaviruses. Priming of caulimovirus reverse transcription uses a tRNA, just as LTR retrotransposons and retroviruses do.

The Evolution of Retroelements

From the above survey, it is evident that there is a wide variety of genetic elements which use reverse transcriptase for their propagation in a broad spectrum of living

organisms from bacteria to man. I said at the outset that we believe the process of reverse transcription to be evolutionarily ancient. Can we assemble all the known manifestations of reverse transcription and the genetic elements involved with this process into an evolutionary tree? In some cases, this is quite easy (Fig. 7). Retroviruses and LTR retrotransposons are obviously related and the more complex gene structure of the former, plus phylogenetic comparisons of the DNA sequences of these elements (Temin, 1980; Xiong and Eickbush, 1990) suggests strongly that LTR retrotransposons were the ancestors of retroviruses (Fig. 7). Judged by sequence homology and structural similarity, the most likely immediate progenitor of retroviruses was a *gypsy* group LTR retrotransposon. *Ty1-copia* group retrotransposons, with their simpler gene structure, probably arose before the *gypsy* group, though whether they were the direct ancestor is unclear.

The hepadnaviruses and caulimoviruses are more difficult to fit into this picture. Phylogenetic analysis suggests that caulimoviruses evolved from *gypsy* group LTR retrotransposons (Doolittle *et al.*, 1989; Xiong and Eickbush, 1990), but hepadnaviruses are highly diverged from both groups. Temin has proposed that both viruses evolved from retroviruses by loss of the ability of the extrachromosomal DNA to integrate into the host chromosome (Temin, 1989). Xiong and Eickbush suggest that hepadnaviruses arose from a recombination event between a pre-existing RNA virus and a primitive retrotransposon while caulimoviruses derived in a similar manner from *gypsy* group retrotransposons. Both models are plausible but the latter is more likely, at least in the case of hepadnaviruses, which have a priming mechanism for the initiation of replication which differs from retroviruses and resembles some RNA viruses, such as poliovirus.

What about the more primitive retroelements? Non-LTR retrotransposons may be the ancestors of LTR retrotransposons, because of their simpler construction, less sophisticated transposition mechanism and ubiquity in the eukaryotes (Doolittle *et al.*, 1989; Xiong and Eickbush, 1990). The evolutionary status of the other elements

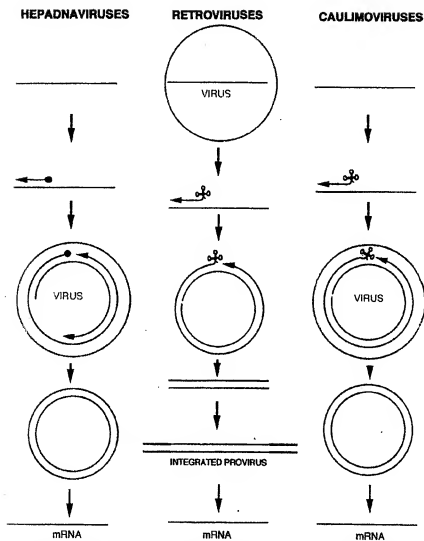


Fig. 6. The life cycles of hepadnaviruses, retroviruses and caulimoviruses compared. DNA is shown in black, RNA in red and protein in green. Arrows refer to the direction of synthesis of DNA by reverse transcriptase.

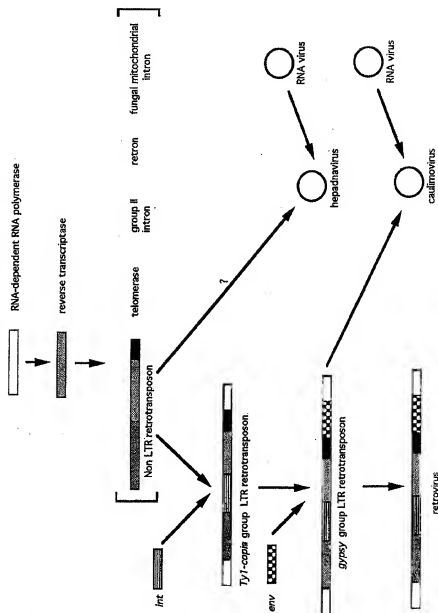


Fig. 7. A possible evolutionary history of retroelements. The genes are designated as in Fig. 5. The relative phylogeny of non-LTR retrotransposons, telomerases, group II introns and retrons is unsure.

mentioned here is still very unclear (Doolittle *et al.*, 1989; Xiong and Eickbush, 1990). Sequence comparisons between the reverse transcriptases argue that retrons and the fungal mitochondrial plasmids are grouped together, with group II introns being the nearest relatives to these sequences and non-LTR retrotransposons the next nearest. Additionally, some non-LTR retrotransposons transpose to the telomeric regions of *Drosophila* chromosomes (Biessman *et al.*, 1992; Levis *et al.*, 1994) suggesting an evolutionary link with telomerases. All this implies that the best candidate for the progenitor of all these elements belongs to an *ad hoc* group containing non-LTR retrotransposons, retrons, telomerases, fungal mitochondrial plasmids and group II introns, though it is impossible to say which, if any, came first.

Two properties unite virtually all retroelements, suggesting that they are fundamental to these elements and were present at their genesis. Firstly, virtually all reverse transcriptases prime the synthesis of new DNA from an RNA molecule with abundant secondary structure which is strongly associated with the enzyme. In the large majority of cases this is a tRNA. tRNAs themselves play a central role in living systems as the key component in the conversion of RNA to protein. RNA was probably the original genetic material and transfer RNAs and reverse transcriptase are the fundamental components of the machinery needed to synthesize DNA and protein, respectively, from RNA. The close association between the two in most retroelements to this day is striking and seems to this author a potent argument for this model of early evolution.

The second property uniting retroelements is their lack of any obvious advantage to their cellular hosts. With the single exception of telomerases, all reverse transcriptases are involved in the propagation of genetic elements which are not involved in the day-to-day functioning of cells. In fact, some retroelements are dangerous parasites (retroviruses, caulimoviruses and hepadnaviruses). Thus, even though their origin probably lies at the dawn of cellular life, these elements have remained aloof from the business of enabling a cell to survive and replicate in an environment.

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EXHIBIT 2

GENOME RESEARCH

The Unusual Phylogenetic Distribution of Retrotransposons: A Hypothesis

Jef D. Boeke

Genome Res. 2003 13: 1975-1983

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Review

The Unusual Phylogenetic Distribution of Retrotransposons: A Hypothesis

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Retrotransposons have proliferated extensively in eukaryotic lineages; the genomes of many animals and plants comprise 50% or more retrotransposon sequences by weight. There are several persuasive arguments that the enzymatic lynchpin of retrotransposon replication, reverse transcriptase (RT), is an ancient enzyme. Moreover, the direct progenitors of retrotransposons are thought to be mobile self-splicing introns that actively propagate themselves via reverse transcription, the group II introns, also known as retrointrons. Retrointrons are represented in modern genomes in very modest numbers, and thus far, only in certain eubacterial and organelle genomes. Archaeal genomes are nearly devoid of RT in any form. In this study, I propose a model to explain this unusual distribution, and rationalize it with the proposed ancient origin of the RT gene. A cap and tail hypothesis is proposed. By this hypothesis, the specialized terminal structures of eukaryotic mRNA provide the ideal molecular environment for the lengthening, evolution, and subsequent massive expansion of highly mobile retrotransposons, leading directly to the retrotransposon-cluttered structure that typifies modern metazoan genomes and the eventual emergence of retroviruses.

The Ancient Origin of Reverse Transcriptase

There are two arguments for an ancient origin of RT. The first is theoretical and is based on the now widely accepted proposal that an RNA world preceded the form of biology with which we are familiar, the DNA world. Darnell first articulated that RT must have been present during the time of the transition between these two worlds, and therefore, must be considered ancient (Darnell and Doolittle 1986; Fig. 1). The second argument is based on the fact that RT genes are very broadly distributed among the branches of the tree of life, and have largely (but not entirely) descended vertically by descent from an ancestral RT gene (Doolittle et al. 1989; Xiong and Eickbush 1990; Eickbush 1994; Malik et al. 1999). Furthermore, the RT gene has seemingly reinvented itself in multiple and diverse forms (Boeke and Stoye 1997). In addition to the familiar retroviruses, there are pararetroviruses, which package DNA, but replicate by reverse transcription, two major classes of retrotransposons (described in the following section), as well as a more bizarre group of elements found in bacteria and organelle genomes, and hence, referred to as the prokaryotic group. The discovery of RTs in bacteria, first in the form of msDNA (short for multicopy single-strand DNA) or retron elements (Yamanaka et al. 2002) and later in the form of retrointrons (Belfort et al. 2002), provided dramatic evidence in favor of an ancient origin for RT.

The highly diverse tree of retroelements can be rooted in the prokaryotic group of elements (Eickbush 1994). The prokaryotic group includes three types, that is, retrons, retroplasmids, and retrointrons. Retrongs are RT genes that produce an unusual branched structure called msDNA made by reverse transcription of a precursor RNA primed from an internal guanosine residue—unlike other retroelements, they have no known function or ability to mobilize autonomously (Yamanaka et al. 2002). Thus far, they have been found only in a very limited subset of bacteria. Retroplasmids are known only from the mitochondria of certain fungi, replicate by reverse transcription, and exist in both circular and linear (hairpin) forms (Kulper and Lambowitz 1988; Walther and Kennel 1999). The retrointrons, or group II introns, mobilize

ize or retrohome to empty target sites (unspliced versions of their host genes) via a very unconventional mechanism. The excised intron lariats insert into double-stranded target DNA (copies of the DNA containing the flanking exons but lacking the intron) by reversal of the normal splicing reaction, probably aided by the maturase activity of the RT proteins encoded by these elements. They are then converted into DNA by use of a target-primed reverse transcription (TPRT) mechanism similar to that used by non-LTR retrotransposons (Zimmerly et al. 1995a,b; Yang et al. 1996). Priming is facilitated by the action of a small endonuclease domain of the RT that cleaves the intact strand of the double-stranded target DNA.

Several independent arguments strongly suggest that the prokaryotic group of RT sequences is ancestral to the RT sequences of retrotransposons and retroviruses. Counterarguments to each of these proposals exist, but as a group, these proposals are compelling. (1) It is a simple evolutionary paradigm that things evolve progressively from a simple state to an ever more complex one. Retrongs, retroplasmids, and retrointrons all encode a single RT protein, often with only that enzymatic activity, whereas retrotransposons and retroviruses always encode multiple enzyme activities and usually encode multiple separate proteins. These additional activities, which include proteases, zinc finger domains, at least three distinct types of endonucleases, and integrase, appear to have been recruited from eukaryotic host genomes at multiple times in evolution, probably using the same types of mechanisms used by retroviruses when they pick up cellular oncogenes (Telesnitsky and Goff 1997). A widely accepted extension of this simple argument is that the retroviruses and pararetroviruses evolved from LTR retrotransposons by acquiring new proteins conferring the ability to efficiently leave and re-enter host cells, also known as horizontal transfer or lateral transfer (Doolittle et al. 1989). (2) The RT of one member of the prokaryotic group has the ability to perform primer-independent synthesis, similar to RNA polymerase, the presumed ancestor of RT (Wang and Lambowitz 1993). (3) The RT sequences of the prokaryotic group are the most similar to the sequences of the presumed ancestral outgroup of sequences, the RNA-directed RNA polymerases (RdRPs). Non-LTR retrotransposons, LTR retrotransposons, and retroviral RTs are progressively less closely related to RdRP sequences (Eickbush 1994). (4)

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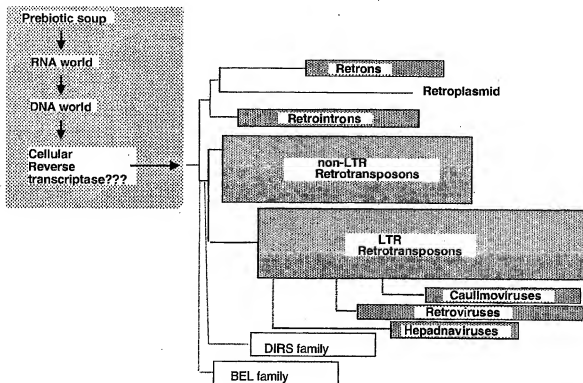


Figure 1 An ancient origin for reverse transcriptase. An early origin of a cellular reverse transcriptase is posited by the RNA world hypothesis (Darnell and Doolittle 1986) (left). The widespread existence of RT genes in prokaryotes, in eukaryotes, and their great molecular diversity (represented by the size of the boxes at the tip of each branch) also suggests an early origin for the RT gene. Tree diagram adapted from Eickbush (1994).

The sequence of telomerase, a specialized RT considered by many to represent an ancient eukaryotic enzyme, clusters with prokaryotic and non-LTR retrotransposon RT sequences (Eickbush 1997; Nakamura and Cech 1998).

Two Types of Retrotransposons That Mobilize by Distinct Mechanisms

The retrotransposons can be divided into two major groups, the non-LTR and the LTR retrotransposons. The mechanisms of these two types of retroelements are summarized briefly here and in Figure 2. In addition, two smaller retrotransposon families, the DIRS1 (Goodwin and Poulter 2001) and BEL (Malik et al. 2000) groups, appear to be distinct, but are much less widely distributed, and thus, will not be discussed further here.

Of the two major retrotransposon classes, the non-LTR retrotransposons, are less well understood mechanistically, but nevertheless, a good outline of the process exists (Kazanian and Moran 1998). The element mRNAs are translated in the cytoplasm, producing one or two proteins. One of these is a polyprotein with at least two critical activities, an endonuclease, and an RT. Most of the non-LTR elements also encode an RNA chaperone, whose role remains unclear. However, the endonuclease/RT protein is thought to bind the element RNA to form an RNP complex, which then enters the nucleus. This complex then acquires a host DNA target, in which a nick is made by the endonuclease. In a remarkable target-primed reverse transcription (TPRT) process, the 3' OH of the cleaved target DNA primes reverse transcription of the element RNA, at or near the 3' poly(A)

end. The mechanism of the cutting of the second strand and second-strand synthesis is less well understood, but may well be symmetrical with the first, involving a second round of TPRT with the newly made DNA strand serving as template.

LTR retrotransposons move via a mechanism quite similar to that used by retroviruses. Generally, two primary protein products are made, corresponding to retroviral Gag (coat proteins) and the readthrough product Gag-Pol (RT and other enzymes). The Gag proteins together with two RNA molecules are assembled into a virus-like particle (VLP). This encapsidation may serve to further protect the element's genomic RNA molecules from degradation. Reverse transcription occurs in the VLP, and is primed by a cellular tRNA (Chapman et al. 1992) or retrotransposon RNA fragment (Levin 1995). The initial product of the RT reaction, minus strand strong-stop DNA, is transferred to the 3' end of the RNA in a critical step that leads to subsequent completion of the minus strand DNA synthesis. If even relatively small amounts of RNA were lost exonucleolytically from the 5' or 3' end during this process, retrotransposition would fail. Several additional steps similar to those used by retroviruses, including a second priming event and strand transfer, lead to the final product of reverse transcription, a double-stranded DNA (Boeke and Stoye 1997; Telesnitsky and Goff 1997). RNA integrity is important for this process, which can take several hours to complete; however, a recombination-like template switching process can bypass damage to the element's RNA. The resulting DNA, together with the integrase protein (processed previously by an element-encoded protease from the RT precursor protein Gag-Pol)

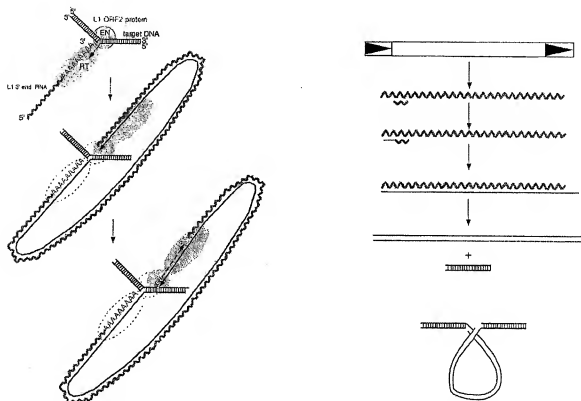


Figure 2 Retrotransposition mechanisms. The lifecycles of non-LTR (left) and LTR retrotransposons are outlined. Wavy lines are RNA molecules; thin black lines are cDNA strands.

is transported to the nucleus, where it inserts via a transesterification reaction very similar to that used by DNA transposons (Mizuuchi and Baker 2002).

Modern-Day Distribution of RT Genes

Faced with the assumption that RT is an ancient enzyme, it becomes difficult to explain the modern-day distribution of RT genes in the three kingdoms of life, Eubacteria, Archaea, and Eukarya. The majority (67%) of sequenced eubacterial species lack a detectable RT gene in their genome (Fig. 3). For those species of eubacteria that do contain RT genes, they mostly contain only one or two RT genes. The great majority of Archaea lack recognizable RTs altogether; the only exception to this trend, *Methanosarcina*, has a very large genome thought to have been formed by the incorporation of a large segment of a eubacterial genome as a late lateral transfer event in its evolution (Deppenmeier et al. 2002). This species contains a set of retrotransposons similar to those found in eubacteria (Dai and Zimmerly 2003). In contrast, RT genes are found in virtually all eukaryotic genomes, and are generally found in 20 to >500,000 copies per genome. Even when adjusted for genome size, eukaryotes contain significantly more RT genes. Virtually all of these are non-LTR and/or LTR retrotransposons. In a recent grand synthesis, Bushman poetically described eukaryotic genomes as "genes floating on a sea of retrotransposons" (Bushman 2002), although an astute reviewer of this work points out that genes do not float, or else gene order colinearity would not be observed in genomic comparisons. Some well-known extreme examples of this include the

human genome, ~1,000,000 non-LTR retrotransposons, SINES, and endogenous retroviruses (Smit 1996) and the maize genome, estimated to contain ~200,000 copies of intact retrotransposons (SanMiguel et al. 1996; J. Bennetzen, pers. comm.). It is the abundance of retroelements that largely explains the C-value paradox in most metazoans. What led to such an abundance of RT genes?

It could be argued that the observed discrepancy is a simple consequence of genome streamlining in bacterial genomes. Although there is no doubt that streamlining is a major evolutionary force in both eubacteria and Archaea, one can consider as a control for the above conclusion the distribution of DNA transposons among the three kingdoms. DNA transposons are found in almost all eubacterial and archaeal genomes and typically are found between 10 and 100 copies. They are also found in eukaryotes, but have a somewhat spottier distribution there, being quite well represented in certain groups (*Drosophila*, *Caenorhabditis*, *Bassoon*), but notably absent from others (*Saccharomyces*, *Schizosaccharomyces*).

The dramatic discrepancy in retroelement distribution between prokaryotes and eukaryotes strongly suggested to me that there was some special feature(s) of being eukaryotic that represented a permissive state for RT and allowed the evolution and proliferation of retrotransposons.

The Evolution of Eukaryotes and Their Retroelements

The release of numerous eubacterial, Archaeal, and eukaryotic genome sequences has provided extensive fodder for models of how eukaryotes evolved. It is clear that we eukaryotes contain a

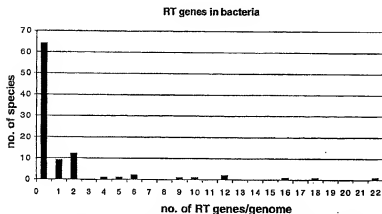


Figure 3 Bacterial genomes contain very few RT genes. A total of 96 completely sequenced bacterial genomes were searched by BLASTp on the comprehensive microbial resource at www.tigr.org. The two queries used were the LtrA RT from a *Leptobacillus lactis* group II intron (Q57005) and a retro RT from *Escherichia coli* (P23070). The number of BLAST hits with an E-value <0.001 was tabulated for both queries, and the higher number was taken as the measure of RT gene number (visual inspection showed that this modestly inflated the number of RT genes as some of the low-scoring hits were false positives).

mixture of genes descended from Archaeal and eubacterial ancestor cells (Woese et al. 1990; Margulis 1996). The precise sequence of events in the evolution of eukaryotes has been debated hotly, but a consensus is developing about the major events that must have occurred. This consensus view will be recounted here briefly.

Archaea and eubacteria were two ancient lineages of cells that had evolved distinct mechanisms of transcription and DNA replication, among other things, but otherwise shared the fundamental properties of being unicellular heterotrophs. Symbiogenesis of eubacterial cells (the progenitor of the mitochondrion) and Archaeal cells ultimately led to a proto-eukaryote containing a eubacterial endosymbiont. This may have begun as a casual or accidental symbiosis, but at some point, provided some important selective advantage. Several other events followed, probably involving an additional cycle(s) of acquiring additional genomes via consumption (Taylor 1974), as well as the acquisition of a number of other distinctive eukaryotic features, which will be considered separately in the next section. These events gave rise to a primitive eukaryote with the recognizable nuclear genome and mitochondrial genome, each in a membrane-bounded compartment. Acquisition of an additional photosynthetic bacterium by consumption led to a plant lineage, but for simplicity, this will not be considered further here. Because the modern eubacteria contain RT genes and the Archaea largely lack them, I will make the fairly arbitrary assumption that the same was true at the dawn of eukaryotes. The eubacterially derived endosymbiont(s) slowly transferred its genes to the nucleus of the primitive eukaryotic cell, becoming ever more dependent on its host. Remarkably, this process of gene transfer from mitochondria to nucleus is functional in modern-day yeast cells, in which the transfer of mitochondrial gene segments to the nucleus can be observed experimentally (Thornton et al. 2002). Through this process, RT genes present as retrointons would be transferred readily to the nucleus through this passive and stochastic process. Movement of retrointons via homing to near-cognate sites might well have led to a proliferation of introns and the evolution of the splicing apparatus as an intron-removal mechanism. The stage was set for the evolution of retrotransposons. What specific features of eukaryotic cells made this possible?

The Nuclear Membrane

The existence of a nuclear membrane would appear to be an impediment and not a help to the evolution of retrotransposition. The translation process occurs outside of the nucleus, whereas transposition happens inside, and therefore, retrotransposons have evolved transport mechanisms to overcome these barriers. Therefore, the existence of the nuclear membrane is inhibitory to successful retrotransposition.

Linear Chromosomes

The transition to linear chromosomes from the presumed ancestral circular state may well have provided an early opportunity for an RT gene to make itself indispensable to its host by acquiring the ability to lengthen telomeres, leading to the enzyme telomerase, still the major mechanism for telomere formation in modern eukaryotes (Nakamura and Cech 1998), providing an elegant solution to the end-replication problem posed by the termini of linear DNA molecules. However, this would provide a niche for but a single copy of RT. Also, a compelling case can be made that telomerase was a relatively late acquisition, by devolution of a retrotransposon (Pardue et al. 1997), as telomeres could, in principle, solve the end-replication problem via the formation of T-loops (Griffith et al. 1999). Thus, linear chromosomes per se do not provide a compelling opportunity for the evolution of retrotransposons.

Introns

As argued above, RT may have played an important role in the widespread accumulation of introns in primitive cells, although the timing of this event has also been the subject of much debate (Gilbert and Glynn 1993; Logsdon Jr. and Palmer 1994; Stoltzfus 1994; Logsdon Jr. 1998; Simpson et al. 2002). However, the simple existence of these introns did not confer any special selective advantage on RT genes. Rather, the proliferation of introns may well be a consequence of a permissive RNA environment that allowed them to mobilize more readily in the genome.

Sex and Diploidy

Donal Hickey (Hickey 1993) and Tim Bester (Bester 1999) have provided eloquent arguments that the evolution of sex and diploidy provides an opportunity for mobile elements to invade host species and march inexorably to fixation in the host genome, providing they do not decrease the fitness of their host >50%. However, this argument applies to both retrotransposons and DNA transposons, and thus, is insufficient to explain the selective amplification of retrotransposons in eukaryotes.

RNA Processing Machinery

The physical separation of the processes of transcription and translation and changes in gene organization (perhaps the consequence of the nascent eukaryal nuclear genome being bordered with fragments of its endosymbiont guest DNA), and other factors, led to important changes in the way RNA was metabolized in eukaryotic cells. The major changes were the compartmentalization of single-coding regions (by and large) into stereotypical mRNA structures punctuated by a 5' cap structure and 3' poly(A) tail (Fig. 4). Not only are the structures of these mRNAs prominent uniquely in eukaryotic cells, but they also coordinate to play a critical role in eukaryotic translational ini-

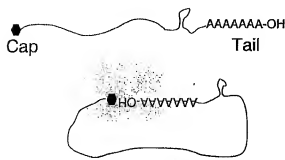


Figure 4 The cap and tail structure of eukaryotic mRNA.

tation; the 5' cap and 3' poly(A) interact in the cytoplasm, effectively circularizing the RNA.

Other Factors

There are likely to be many factors that control the proliferation of transposable elements of all types in eukaryotes. For example, organisms such as yeasts and *Drosophila*, with smaller genomes, tend to have much higher recombination rates, and these organisms carry lighter transposon burdens. Ergo, it can be argued that such high recombination rates are inconsistent with explosive types of transposon amplification, such as has been seen in humans and maize. Very high transposon copy numbers could cause extensive secondary damage to highly recombination-proficient genomes. Similarly, diverse mechanisms controlling the copy number of certain transposons' activity, such as cosuppression of Ty1 elements in yeast (Jiang 2002) and RNAi in many eukaryotes (Ketting et al. 1999), play important roles in controlling transposon copy numbers. Whereas such factors are undoubtedly of considerable importance in determining transposon copy numbers in individual species, they do not help to explain the general trend observed that eukaryotes tend to have very high retrotransposon copy numbers relative to prokaryotes.

The cap and tail hypothesis proposes that this unique terminal structure created three special molecular opportunities for the evolution of retrotransposons. First, these termini created a very stable long-lived genomic RNA freed from the necessity to be highly folded. Second, this RNA stability facilitated the recombinational acquisition of additional host gene modules needed for the formation of retrotransposons much more likely; the long mRNAs typical of retrotransposons and retroviruses were protected from destruction by exonucleases. Third, these terminal RNA structures provided precise punctuation marks defining the retrotransposon termini and facilitating their reproduction without the loss of even a single terminal nucleotide. These traits set the stage for the evolution of elaborate and precise processes of reverse transcription evolved by retrotransposons.

Why Did Eukaryotes Evolve Caps and Tails?

A number of theories have been advanced as to the evolution of the cap and tail. Extensive work on the molecular biology of translation has shown that the 5' cap and 3' tail structures are directly required for initiation of translation in eukaryotes. Additionally, both RNA structures are protective against terminal degradation of the RNA. In particular, the protective role of the 5' cap is revealed by the eukaryotic mRNA degradation pathway; this process occurs in three steps, (1) 3' deadenylation, leading to (2) decapping, followed by (3) 5' → 3' exonuclease action (Tucker and Parker 2000). Although 3' exonucleases are found in eukaryotic cells, they appear to play more specialized roles in mRNA

stability, such as nonstop decay and the destabilization of specific mRNAs (van Hoof and Parker 2002).

Polyadenylation occurs in all three kingdoms of life, although it only affects a subset of mRNAs in bacteria, and actually stimulates mRNA breakdown in prokaryotes (Stege 2000). Thus, certain components of the polyadenylation machinery predated the evolution of eukaryotes, and it appears that poly(A) simply acquired new functions in eukaryotes. In the literature and in discussions with colleagues, I've become aware of three theories regarding selective pressures leading to a need for a 5' cap. The first theory is that the compartmentalization of transcription leads to extensive opportunities for potentially inhibitory RNA folding prior to translation—potentially, such mRNA hairballs could occlude internal Shine Delgarno initiation sequences, whereas a terminal cap structure could more readily be recognized, like the end of a ball of yarn (Hershey and Merrick 2000). A second theory is that the complex nature of RNA processing in eukaryotes could lead to large numbers of misprocessed RNAs. Expression of inappropriately processed RNAs could lead to the expression of deleterious dominant negative protein fragments for example. Obviously, there are special pathways such as Non-sense-mediated decay (Frischmeyer and Dietz 1999; Gonzalez et al. 2001) and Nonstop decay, which deal with some of the RNA quality control issues raised by the existence of potentially inaccurate splicing machinery. However, a third type of proofreading is conferred by the obligatory circularization of mRNA during translational initiation—any RNA lacking intact 5' or 3' ends will not be translated (R. Green, pers. comm.).

Finally, Stewart Shuman has proposed that the cap arose to protect the RNA from 5' exonuclease action, and that the latter activity represented a type of primitive immunity against RNA viruses (Shuman 2002). Thus, the Xnlp 5' exonuclease may have arisen in response to genomic RNA invaders, and the capping machinery evolved in parallel to protect endogenous cellular mRNAs. It is clear that eukaryotic cells evolved a series of different immunity mechanisms against invading RNA genomes, including the interferon system (Kumar and Carmichael 1998) and RNAi (Ketting et al. 1999). Needless to say, if this scenario is correct, the primitive immunity conferred by 5' exonuclease was quickly evaded by viruses that acquired caps by various nefarious means or evolved IRES elements that bypassed the cap requirement (Shuman 2002). Nevertheless, it would appear that the acquisition of the cap/5' exo strategy paradoxically set the stage for the evolution of a collection of internal genome invaders of eukaryotes, and eventually, retroviruses.

An interesting difference between bacteria and eukaryotes that may be related to differential RNA stability is the ability of eukaryotes to produce significantly longer proteins, such as the long polypeptides encoded by retrotransposons. Interestingly, a survey of bacterial genomes (Fig. 5) shows that bacteria, on average, encode shorter proteins than eukaryotes. This discrepancy becomes particularly acute when the longest ORFs are examined. The longest ORF in *Escherichia coli* K12, a putative invasin at 2383 codons, is less than half the length of the longest *Saccharomyces cerevisiae* ORF, the *MDN1* gene at 4910 codons, and pales in comparison to human titin at 27,118 amino acids, encoded by an astonishingly long 82-kb mRNA (Labat and Kolmerer 1995). This limit to ORF size does not represent an absolute expression block in bacteria, as some very large ORFs encoding nonribosomal polypeptide and polypeptide synthases have been discovered in various bacterial species. It is possible that the simple lifestyle of prokaryotes generally requires shorter proteins than the complex lifestyle of eukaryotes. The evolution of a more stable mRNA structure in eukaryotes may well have contributed to the evolution of much greater potential protein structure complexity in general in eukaryotes.

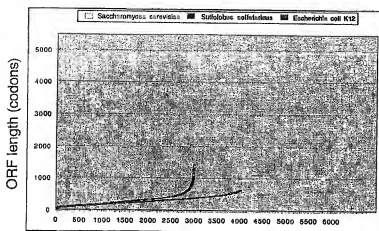


Figure 5 Bacteria and Archaea encode smaller proteins than eukaryotes. The number of codons in each ORF for the indicated organisms was sorted in *desc*, and a point was plotted for each protein. It can be seen readily that both the mean length and total length of the eukaryotic proteins are significantly higher than those of both the eubacterial and archaeal species. Results are typical (data not shown).

Bacterial Are RNA-Hostile

Recent work on the degradation of bacterial mRNAs has elucidated the basic molecular mechanisms, which are quite different from the eukaryotic mechanism (Table 1). In summary, eubacteria like *E. coli* degrade their RNAs through the combined effects of multiple endonucleases and 3' exonucleases; many of the relevant activities are organized in degradosomes (Steeg 2000). One of the well-studied endonucleases, RNase E, nicks unstructured RNA regions adjacent to structured regions. Whereas the products of such nicking are not necessarily excellent direct substrates for the 3' exonucleases, addition of a 3' poly(A) tail creates an opportunity to initiate the degradation process by the degradosome; hence, mRNA polyadenylation leads to degradation in eubacteria.

If the cap and tail hypothesis is correct, it makes a number of predictions—for example, intact long RNA molecules should be difficult to detect in bacteria. It has long been known that it is extremely difficult to detect bacterial mRNAs by Northern blotting, and typical measurements of bacterial RNA half-lives range from seconds to minutes—far shorter than the half-lives of their eukaryotic counterparts, even when the mean mRNA half-life is adjusted for the cell generation time. (Fig. 6). Only a single value for average mRNA half-life is available from an Archaeal species, *Sulfolobus solfataricus*, which is among the slower-growing Archaea (some Archaea have fast doubling times similar to those of eubacteria), and its RNA half-life value is intermediate between eubacteria and *S. cerevisiae*, a eukaryote with a relatively short mRNA half-life (Bint et al. 2002). Examination of the mRNA degradation components encoded in eubacterial, Archaeal, and eukaryotic genomes shows that the eubacteria and Archaea share most of the same genes. Homologs of RNase E, RNase II, and polynucleotide phosphorylase are readily found by BLAST searching against Archaeal genomes. In contrast, Xrn1p homologs and capping enzyme homologs are absent from eubacteria and Archaea, but are common to all eukaryotes (Anantharaman et al. 2002). Furthermore, like eubacteria, Archaea organize at least some of their genes in operons, and use Shine-Delgarno sequences to guide ribosomes to their initiation sites, at least in

some mRNAs (Shuman 2002), suggesting that translational initiation mechanisms in Archaea are more similar to those in eubacteria than in eukaryotes.

Eubacterial Retroelements Have Small, Highly Structured RNAs With Occluded 3' Ends

A second prediction of the cap and tail model is that those retroelements that are found in eubacteria and Archaea will exhibit genomic features suggestive of protection against RNA degradation, such as short length, extensive secondary structure, and occluded 3' ends. The two major classes of eubacterial retroelements display just these features. Retrointron RNA genomes are much shorter than retrotransposons and retroviruses, typically extending only 1–2 kb long versus 4–8 kb or more for typical retrotransposons and 10 kb or more for typical retroviruses. They are highly folded, their 5' end is occluded via a 2'-5' linkage and, moreover, they are always found in the form of a highly specific RNP, in which the RT-maturase protein is tightly bound to the intronic RNA. Importantly, the 3' terminus of these molecules consists of a series of Watson-Crick base pairs at the base of the domain VI stem of the intron, followed by two or three unpaired bases that can form a tertiary interaction with an internal segment in the intron (y/y' sequences; Bonen and Vogel 2001). Similarly, the retroon genome consists of a small, highly folded molecule in which the 3' end of the RNA component is base paired to the 3' end of the DNA component (Yamanaka et al. 2002).

Retrotransposon RNAs Are Capped and Polyadenylated

Nearly all retrotransposon RNAs contain caps and poly(A) tails, as do retroviral RNAs. The case is quite clear for LTR retrotransposons and retroviruses; there are many reports of poly(A) at the 3' ends of LTR retrotransposon RNAs, and further evidence for posttranscriptionally added 3' poly(A) tails in LTR retrotransposons can be found readily in EST databases. Capping is more laborious to evaluate, but some studies have found evidence for example, Ty1 mRNA was examined directly and found to be capped (Mules et al. 1998b), as are retroviral RNAs. Because LTR retrotransposons encode proteins required for their own mobility, and these must be translated from their mRNAs, it is extremely likely that all LTR-retrotransposon RNAs are capped.

One of the most important characteristics of non-LTR retrotransposons is that the vast majority of these elements actually encode poly(A) in their DNA. This 3' poly(A) tract defines the element's 3' end; many studies suggest that the 3' poly(A) tract defines the site at which reverse transcription (TRT) initiates (Moms et al. 1996). These poly(A) tails are peculiar in that they are apparently synthesized, at least in part, by RNA polymerase rather than the conventional polyadenylation machinery. However, it is possible that the 3' poly(A) residues might be added post-transcriptionally using conventional polyadenylation. There are a few non-LTR retrotransposons such as the *Drosophila* 1 factor, which terminate not in poly(A), but in a related sequence, (TAA)_n. Clearly, the 3' end of 1 factor RNA is not formed by conventional polyadenylation, but by transcription. Nevertheless, the number of TAA repeats can increase during retrotransposition, suggesting that a mechanism other than conven-

Table 1. RNA Degradation Components Found in the Three Kingdoms of Life

Enzyme	Activity	Distribution*
Prokaryotic		
RNAse E (me)	Endoribonuclease; cuts single-stranded DNA adjacent to structured regions; provides internal access points for degradation components	Eubacteria, Archaea (Eukarya—weak, exosome)
RNAse G (mg)	Endoribonuclease; cuts single-stranded DNA adjacent to structured regions; provides internal access points for degradation components	Eubacteria, Archaea (Eukarya—weak)
RNAse III (mc)	Endoribonuclease; cuts double-stranded DNA; provides internal access points for degradation components	Eubacteria, Archaea (some homologs in Eukarya)
RNAse II (mb)	3'-5' Exoribonuclease	Eubacteria
Polynucleotide Phosphorylase (mpg)	3'-5' Exoribonuclease	Eubacteria, Archaea (some homologs in Eukarya)
Oligoribonuclease (om)	3'-5' Exoribonuclease	Eubacteria, Archaea
Eukaryotic		
Deadenylase (CCR4)	Removes nucleotides from 3' poly(A) during translation (Chen et al. 2002; Tucker et al. 2002)	Eukarya
Decapping enzyme (DCP2)	Removes 5' cap from deadenylated mRNA	Eukarya
Exonuclease (XRN1)	5'-3' exonuclease	Eukarya
Exosome (multiple)	3'-5' exonuclease	Eukarya (prokaryotes, weak)

*Taken from Aparitharaman et al. (2002)

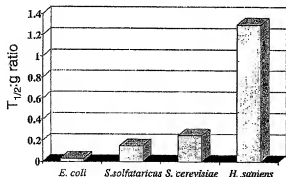
tional polyadenylation leads to the lengthening of the element 3' end, probably slippage by the I factor RT (Pritchard et al. 1988). Interestingly, the I factor 3' sequence can be replaced with poly(A), and the modified elements produce progeny elements with 3' poly(A) tails (Chambeyron et al. 2002). Intriguingly, a significant subset of human L1 elements carry a related (TAAA), repeat in place of poly(A) (Szak et al. 2002). There are a few non-LTR retrotransposons, such as the CR1 element that terminate in a 3' terminal-repeated sequence unrelated to poly(A) (Burch et al. 1993). Presumably, these mRNAs have found another way to be circularized during translation, as they must be translated. Because this type of element lacking a poly(A)-like sequence is rare, I would propose that this is some late evolutionary adaptation. Clearly, the ancestral state of this family of elements is a 3' poly(A) tail.

Capping, however, has not been directly studied in the non-LTR retrotransposons, although the similarity of these elements' RNAs to mRNA strongly suggests that they are capped. There is evidence that the *Drosophila* jockey non-LTR retrotransposon is transcribed by RNA polymerase II, which is that its mRNA synthesis is α -amanitin sensitive (Mizukoshi et al. 1988). All known pol II mRNAs are capped, therefore, non-LTR mRNAs are unlikely

to be exceptions to this rule. Finally, the sequence of the human L1 element provides presumptive evidence for capping. Previous *in vitro* studies have shown that various RTs can readily copy the G residue comprising the cap, in spite of its unusual 5'-5' triphosphate linkage to the mRNA (Hirzmann et al. 1993; Volloch et al. 1995; Mules et al. 1998). The L1 sequence starts with a run of a variable number G residues, which I propose has accumulated through multiple rounds of cap reverse transcription. In support of this exotic idea, the majority of extra single nucleotides accumulated at the 5' junction of experimentally isolated new full-length L1 insertions are G residues, whereas truncated L1 elements do not prefer single-G insertions (Symer et al. 2002; N. Gilbert, S.L. Lutz-Prigge, and J.V. Moran, pers. comm.).

A final exception to the general rule that eukaryotic retrotransposons are capped and polyadenylated is also instructive and supports the model, namely, the case of the *Alu* element and the related SINEs. These unusual elements don't need a cap, because they are not translated, but rely on retrotransposition proteins encoded by other non-LTR retrotransposons. Intriguingly, these elements are polyadenylated through transcription, even though they are transcribed by RNA polymerase III and, hence, are extremely unlikely to interact with the polyadenylation apparatus. However, these pol III transcripts lack a 5' cap. A different mechanism of protection from 5' exonuclease is adopted by these elements; as in the case of eubacterial retroelement 3' ends, *Alu* and related retroelements, as well as the tRNA-derived retroelements, are also highly folded and the 5' end of the RNA is always found in an extensively base-paired structure (e.g., see Sinnett et al. 1992), which would protect it against Xrn1-like 5' exonucleases.

Retrotransposon RNA levels are highly variable and tend to be tissue specific in metazoans, with high levels reached only in the germ line in most cases (Chaboisier et al. 1990; Branciforte and Martin 1994). Naturally, the abundance of retrotransposon RNAs is very strongly correlated with retrotransposition frequency. Because retrotransposition frequencies are set by some complex evolutionary interplay unique to each host/retrotransposon combination, it is not surprising that there is great variability in retrotransposon RNA levels. Nevertheless, there are some very dramatic cases of very high retrotransposon RNA lev-

**Figure 6.** Average mRNA half-lives of diverse organisms, adjusted for generation time. The data are plotted directly from Bini et al. (2002).

els that provide strong evidence that the cap and tail structure are compatible with high levels of retrotransposon transcript stability. Of note, the *Drosophila* retrotransposon *copia* is so named because of its incredibly copious mRNA (Young and Hogness 1977), and yeast Ty1 mRNA levels are among the most abundant in the yeast cell (Curcio et al. 1990), with Ty1 mRNA visible as a discrete band in poly(A)-selected RNA preparations.

In conclusion, the stable and well-punctuated RNA system was probably critical in allowing eukaryotes to evolve an ever more complex lifestyle, permitting longer more complex proteins and increased molecular diversity through alternative splicing. This same key change probably led to the extensive proliferation of retroelements, including retroviruses, in the many complex guises in which they are found today.

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WEB SITE REFERENCES

www.tigr.org: Comprehensive Microbial Resource at the Institute for Genome Research.

EXHIBIT 3

mine. A single dose of clozapine increases dopamine release in the primate prefrontal cortex, and long-term administration increases basal extracellular dopamine concentration in the prefrontal cortex (21). Although this may not be the only mechanism by which clozapine elicits its effects on PCP-induced cognitive dysfunction, this activation of the dopamine system of the prefrontal cortex may contribute to the ability of clozapine to ameliorate the impairments in our model and, perhaps, in schizophrenia.

Our data show that repeated administration of PCP inhibits basal and stimulated dopaminergic function in the prefrontal cortex of the monkey brain. The deficiency of dopamine in the prefrontal cortex that is induced by repeated administration of PCP is associated with a long-lasting cognitive deficit, which is ameliorated by the atypical therapeutic drug clozapine. These effects are observed long after PCP administration is stopped and thus cannot be attributed to direct effects of the drug. This primate model of dopamine dysfunction in the cortex may provide a paradigm for investigating the pathophysiology underlying neuropsychiatric disorders associated with a primary cognitive dysfunction in the cortex and a dopaminergic deficit in the prefrontal cortex, as is hypothesized in schizophrenia (22). It also may provide a means for evaluating therapeutic agents that are selectively targeted toward alleviating cortical dopamine hypofunction.

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Telomerase Catalytic Subunit Homologs from Fission Yeast and Human

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Catalytic protein subunits of telomerase from the ciliate *Euplotes aediculatus* and the yeast *Saccharomyces cerevisiae* contain reverse transcriptase motifs. Here the homologous genes from the fission yeast *Schizosaccharomyces pombe* and human are identified. Disruption of the *S. pombe* gene resulted in telomere shortening and senescence, and expression of mRNA from the human gene correlated with telomerase activity in cell lines. Sequence comparisons placed the telomerase proteins in the reverse transcriptase family but revealed hallmarks that distinguish them from retroviral and retrotransposon relatives. Thus, the proposed telomerase catalytic subunits are phylogenetically conserved and represent a deep branch in the evolution of reverse transcriptases.

Telomerase is a ribonucleoprotein enzyme responsible in most eukaryotes for the complete replication of chromosome ends, or telomeres (1). Its RNA subunit provides the template for addition of short sequence repeats typically 6 to 26 nucleotides (nts) to the chromosome 3' end (2). In ciliated protozoa and yeast, telomerase is regulated and the average telomere length is maintained (3). In most human somatic cells, however, telomerase activity cannot be detected and telomeres shorten with successive cell divisions (4). Telomerase activity

reappears in immortalized cell lines and in about 85% of human tumors, which has led to studies of the usefulness of telomerase for cancer diagnostics and therapeutics (5, 6).

Telomerase RNA subunits have been identified and analyzed in ciliates, yeast, and mammals (2, 7), but the protein subunits have been elusive. In *Tetrahymena*, two telomerase-associated proteins (p80, p95) have been described (8), and p80 homologs have been found in humans and rodents (9); the presence of catalytic active site residues in these proteins has not been

established. Purification of telomerase from the ciliate *Euplotes aediculus* yielded two proteins, p123 and p43 (10), that appear unrelated to p80 and p95 (11). p123 contains reverse transcriptase (RT) motifs and is homologous to yeast Est2 (Ever shorter telomeres) protein (11), which is essential for telomere maintenance in vivo (12). The RT motifs of Est2p are essential for telomeric DNA synthesis in vivo and in vitro (11, 13), supporting the conclusion that Est2p and p123 are the catalytic subunits of telomerase. The question remained whether there are two telomerases in biology, one based on p80- and p95-like proteins and one on p123/Est2p-like proteins.

To determine if Est2p/p123 is conserved among eukaryotes, we searched for homologs in the fission yeast *S. pombe* and humans. Polymerase chain reaction (PCR) amplification of *S. pombe* DNA was carried out with degenerate-sequence primers designed from the *Euplotes* p123 RT motifs B' and C. Of the four prominent products generated, the ~120-base pair (bp) band encoded a peptide sequence homologous to p123 and Est2p. Using this PCR product as a probe for colony hybridization, we identified two overlapping clones from a genomic library and three from a cDNA library (14). None of the three cDNA clones was full length, so we used RT-PCR to obtain the NH₂-terminal sequences (15). This putative telomerase reverse transcriptase gene, *trt1*⁺, encoded a basic protein with a predicted molecular mass of 116 kilodaltons (kD) (Fig. 1A). The sequence similarity to p123 and Est2p was especially high in the seven RT motifs (Table 1) and in motif T (Telomerase-specific) (Fig. 2). Fifteen introns, ranging from 36 to 71 bp, interrupted the coding sequence. All had consensus splice and branch site sequences (16).

If *trt1*⁺ encodes the telomerase catalytic subunit in *S. pombe*, deletion of the gene would be expected to result in telomere shortening and perhaps cellular senescence as seen with the *est2* mutants in *S. cerevisiae* (11, 13). To test this, we created two deletion constructs (Fig. 1A), one removing motifs B' through E in the RT domain, and the second deleting 99% of the open reading frame (ORF). Haploid cells grown from both types of spores showed progressive telomere shortening to the point where hybridization to telomeric repeats became al-

most undetectable (Fig. 1B). Senescence was indicated by (i) reduced ability of the cells to grow on agar, typically by the fourth streak-out after germination; (ii) the appearance of colonies with increasingly ragged edges (Fig. 1C); and (iii) the increasing fraction of elongated cells (Fig. 1D). When individual enlarged cells were separated on the dissecting microscope, the majority underwent no further division. The same *trt1*⁺ cell population always contained normal-size cells that continued to divide but frequently produced nondividing pro-

eny. The telomerase-negative survivors may use a recombinational mode of telomere maintenance as documented in budding yeast strains with deletions of telomere-replication genes (12, 17).

A candidate human p123/Est2p/Trt1p homolog was identified by a BLAST search of the EST (expressed sequence tag) database (GenBank AA281296). This EST was the top-ranked match in sequence searches with *Euplotes* p123 ($P = 3.3 \times 10^{-6}$) and *S. pombe* Trt1p ($P = 9.7 \times 10^{-7}$). The human EST was not found in searches with yeast

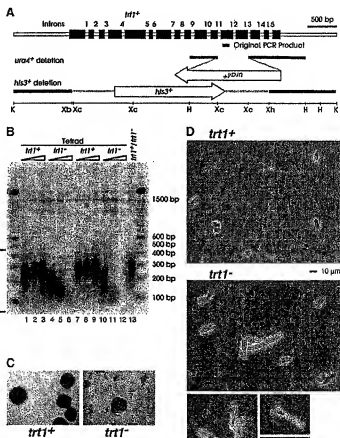


Fig. 1. The gene for the *S. pombe* telomerase protein and phenotypes associated with its deletion. (A) The *trt1*⁺ locus, the location of the ~120 bp PCR product that led to its identification, and the regions replaced by *ura4*⁺ or *his3*⁺ genes in the *trt1*⁻ mutants (K, Kpn I; Xb, Xba I; H, Hind III; Xc, Xco I; Xh, Xho I). (B) Telomere shortening phenotype of *trt1*⁻ mutants. A *trt1*⁺/*trt1*⁻ diploid (28) was sporulated and the resulting tetrads were dissected and germinated on a YES (Yeast Extract medium Supplemented with amino acids) plate (29). Colonies derived from each spore were grown at 32°C for 3 days, and restreaked successively to fresh YES plates every 3 days. A colony from each round was placed in 5 ml of YES liquid culture at 32°C and grown to stationary phase, and genomic DNA was prepared. After digestion with *Apa* I, DNA was subjected to electrophoresis on a 2.3% agarose gel, stained with ethidium bromide to confirm approximately equal loading in each lane, transferred to a nylon membrane, and hybridized to a telomeric DNA probe. The *Apa* I site is located 30 to 40 bp away from telomeric repeat sequences in chromosomes I and II. (C) Colony morphology of *trt1*⁺ and *trt1*⁻ cells. Cells plated on MM [Minimal Medium (29) with glutamic acid substituted for NH₄Cl] were grown for 2 days at 32°C prior to photography. (D) Micrographs of *trt1*⁺ and *trt1*⁻ cells grown as in (C).

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Est2p, but subsequent pairwise comparison of these sequences showed a convincing match. Sequencing of the rest of the cDNA clone containing the EST revealed all eight TRT (Telomerase Reverse Transcriptase) motifs, but not in a single ORF (18). We used the sequence information from this incomplete cDNA clone to isolate an extended cDNA clone from a library of 293 cells, an adenovirus E1-transformed human embryonic kidney cell line (19). This cDNA clone (pGRN121) had a 182-bp insert relative to the EST clone, which increased the spacing between motifs A and B' (18) and put all seven RT motifs and the telomerase-specific motif T in a single contiguous ORF (Fig. 2).

RT-PCR amplification of RNA from 293 cells and from testis each gave two products differing by 182 bp (20). The larger and smaller products from testis RNA were sequenced and found to correspond exactly to pGRN121 and the EST cDNA, respectively.

The relative abundance of hTERT mRNA was assessed in six telomerase-negative mortal cell strains and six telomerase-positive immortal cell lines (21) (Fig. 3). The steady-state level of hTERT mRNA was higher in immortal cell lines with active telomerase (6) than in any of the telomerase-negative cell strains tested. Telomerase activity was more strongly correlated with the abundance of hTERT mRNA than with that of telomerase RNA

(hTERT) (7). In contrast, the abundance of mRNA for the human p80 homolog TP1 (9) did not correlate with telomerase activity (Fig. 3). Thus, while our proposal that hTERT is the catalytic subunit of human telomerase is based mainly on protein structural features

Table 1. Amino acid sequence identity between telomerase reverse transcriptases. Each value is % identity (% similarity in parentheses) based on RT motifs 1, 2, and A through E in Fig. 2C.

	hTERT	SpTtr1p	Est2p
Ea p123	26 (49)	28 (45)	24 (48)
Est2p	28 (46)	27 (48)	
SpTtr1p	30 (47)		

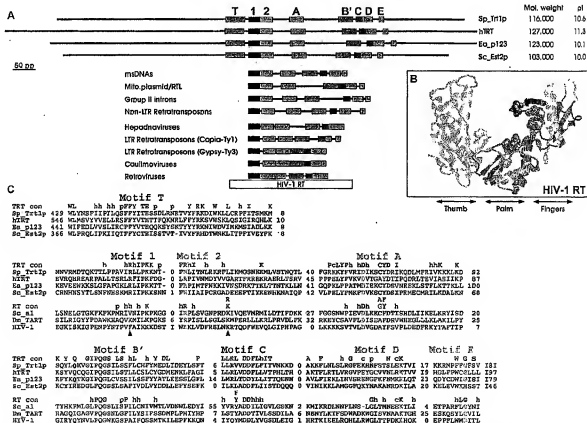


Fig. 2. Structure and RT sequence motifs of telomerase proteins. (A) Locations of telomerase-specific motif T and conserved RT motifs 1, 2, and A through E [24] are indicated by colored boxes. The open rectangle labeled HIV-1 (Human Immunodeficiency Virus) RT delineates the portion of this protein shown in (B). *pt*, isoelectric point. (B) The crystal structure of the p80 subunit of HIV-1 RT (Brookhaven code 1HHV). Color-coding of RT motifs matches that in (A). The view is from the back of the right hand, which allows all motifs to be seen. (C) Multiple sequence alignment of telomerase RTs and members of other RT families (Sc₁, cytochrome oxidase group II intron 1-encoded protein from *S. cerevisiae*; mtDNA; TART, reverse transcriptase from *Drosophila melanogaster*; TART non-LTR retrotransposable element). Boldface residues indicate identity of at least three telomerase sequences in the alignment. Colored residues are highly con-

served in all RTs and shown as space-filled residues in (B). The number of amino acids between adjacent motifs or to the end of the polypeptide is indicated. TRT con and RT con, consensus sequences for telomerase RTs (this work) and non-telomerase RTs (24) (amino acids are designated h, hydrophobic; A, L, I, V, P, F, W, M; p, polar; G, S, T, Y, C, N, Q, c, charged; D, E, H, K, R, F). Red snowflakes show some of the systematic differences between telomerase proteins and other RTs. Red rectangle below motif E highlights the primer grip region discussed in the text. Abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequences of the *S. pombe trt1* gene and the human TRT cDNA (pGRN121) have been deposited in GenBank (accession nos. AF015783 and AF015950, respectively).

(similarity of RT motifs, the T motif, molecular mass > 100 kD, $pI > 10$), the correlation of its mRNA expression level with activity also supports this conclusion.

Sequence alignment of the four telomerase genes revealed features similar to other reverse transcriptases, as well as differences that serve as hallmarks of the telomerase subgroup. The new T motif is one telomerase-specific feature not found in the other RTs examined. Another is the distance between motifs A and B', which is

longer in the TRTs than in other RTs (Fig. 2A). These amino acids can be accommodated as an insertion within the "fingers" region of the structure that resembles a right hand (22, 23) (Fig. 2B). Within the motifs, there are a number of substitutions of amino acids (red arrowheads in Fig. 2C) that are highly conserved among the other RTs. For example, in motif C the two aspartic acid residues (DD) that coordinate active site metal ions (22) occur in the context hxD(hY) in the telomerase RTs compared to (D)Y(h)DD in the other RTs (24). Another systematic change characteristic of the telomerase subgroup occurs in motif E, where WxGxSx appears to be the consensus among the telomerase proteins, whereas hLGGxh is characteristic of other RTs (24). This motif E is called the "primer grip" (23), and mutations in this region affect RNA priming but not DNA priming (25). Because telomerase uses a DNA primer, the chromosome 3' end, it is not unexpected that it should differ from other RTs in this region. Given that the simple change from Mg²⁺ to Mn²⁺ allows HIV RT to copy a small region of a template in a repetitive manner (26), it is tempting to speculate that some of the distinguishing amino acids in the TRTs may cause telomerase to catalyze repetitive copying of the template sequence of its tightly bound RNA subunit.

Using the seven RT domains (Fig. 2C) defined by Xiong and Dickbush (24), we constructed a phylogenetic tree that includes the four telomerase RTs (Fig. 4). The TRTs appear to be more closely related to RTs associated with msDNA (multicopy single-stranded DNA), group II introns, and non-LTR (Long Terminal Repeat) retrotransposons than to the LTR-retrotransposon and viral RTs. The relationship of the telomerase RTs to the non-LTR branch of retroelements is intriguing, given that the latter elements have replaced telomerase for telomere maintenance in *Drosophila*

(27). However, the most striking finding is that the TRTs form a discrete subgroup, about as closely related to the RNA-dependent RNA polymerases of plus-strand RNA viruses such as poliovirus as to retroviral RTs. In view of the fact that the four telomerase genes are from evolutionarily distant organisms—protozoan, fungi, and mammals—this separate grouping cannot be explained by lack of phylogenetic diversity in the data set. Instead, this deep branching suggests that the telomerase RTs are an ancient group, perhaps originating with the first eukaryote.

The primary sequence of hTRT and eventual reconstitution of active human telomerase may be used to discover telomerase inhibitors, which in turn will permit additional testing of the anti-tumor effects of telomerase inhibition. The correlation between hTRT mRNA levels and human telomerase activity shown here indicates that hTRT also has promise for cancer diagnosis. With an essential protein component of telomerase now in hand, the stage is set for more detailed investigation of fundamental and applied aspects of this ribonucleoprotein enzyme.

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- PCR primers were based on motif B' (YARACAAAGGAGATYCCYAGAG) and motif C (NGTATDARDARTARTTCCTTC), where D = G, A, or T; H = T, A, or C; Y = T or C; R = A or G; and N = G, A, T or C. Approximately 10⁶ colonies of an *S. pombe* Hind III-cut genomic library in pGW5 [A. Wright, K. Maundrell, W. D. Hoyer, D. Beach, P. Nurse, *Plasmid* **15**, 156 (1993)] and 10⁶ colonies of an *S. pombe* cDNA library [L. P. Javerzi, G. Cranston, R. C. Allshire, *Nucleic Acids Res.* **24**, 4676 (1996)] were screened.

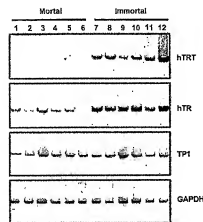
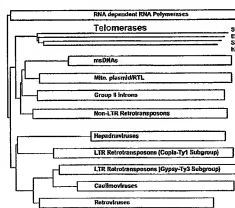


Fig. 3. Expression of hTRT in telomerase-negative mortal cell strains (lanes 1 to 6) and telomerase-positive immortal cell lines (lanes 7 to 12). RT-PCR (21) for hTRT, hTR (human telomerase RNA component), TP1 (telomerase-associated protein related to Tetrahymena p80), and GAPDH (to normalize for equal amounts of RNA template) was carried out on RNA from: (1) human fetal lung fibroblasts GFL, (2) human fetal skin fibroblasts GFS, (3) adult prostate stromal fibroblasts 31YO, (4) human fetal knee synovial fibroblasts HSF, (5) neonatal foreskin fibroblasts BJ, (6) human fetal lung fibroblasts IMR90, (7) melanoma LOX IMV, (8) leukemia U251, (9) NO1 H23 lung carcinoma, (10) colon adenocarcinoma SW620, (11) breast tumor MCF7, and (12) human 293 cells.

Fig. 4. A possible phylogenetic tree of telomerases and retroelements rooted with RNA-dependent RNA polymerases. After sequence alignment of motifs 1, 2, and A through E (178 positions, Fig. 2C) from four TRTs, 67 RTs, and three RNA polymerases, the tree was constructed using the Neighbor Joining method (30). Elements from the same class that are located on the same branch of the tree are simplified as a box. The length of each box corresponds to the most divergent element within that box.



- [illegible]

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Contrasting Genetic Influence of *CCR2* and *CCR5* Variants on HIV-1 Infection and Disease Progression

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Cohort Study (MHCS), San Francisco City Cohort (SFCC),
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The critical role of chemokine receptors (CCR5 and CXCR4) in human monocucleofcy virus type 1 (HIV-1) infection and pathogenesis prompted a search for polymorphisms. In other chemokine receptor genes that mediate HIV-1 disease progression, a mutation (CCR2-64) within the first transmembrane region of the CCR2 chemokine and HIV receptor gene is described that occurred at an allele frequency of 10 to 15 percent among Caucasians and African Americans. Genetic association analysis of five acquired immunodeficiency syndrome (AIDS) cohorts (3003 patients) revealed that although CCR2-64 exerts no influence on the incidence of HIV-1 infection, HIV-1-infected individuals carrying the CCR2-64 allele progressed to AIDS 2 to 4 years later than individuals homozygous for the common allele. Because CCR2-64 occurs invariably on a CCR5-+/- bearing chromosomal haplotype, the independent effects of CCR5-Δ32 (which delays AIDS onset) and CCR2-64 were determined. An estimated 38 to 45 percent of AIDS patients whose disease progresses rapidly (less than 3 years until onset of AIDS symptoms after HIV-1 exposure) can be attributed to their CCR2-+/- or CCR5-+/- genotype, whereas the survival of 28 to 29 percent of long-term survivors, who avoid AIDS for 16 years or more, can be explained by a mutant genotype for CCR2 or CCR5.

The needs of chemokine immunobiology and AIDS pathogenesis has revealed unmet requirements for resolving patterns of HIV disease progression, for defining epidemiologic heterogeneity, and for guiding therapeutic (1-6). Identification of chemokines, RANTES, MIP1 α and MIP1 β , as suppressor factors produced by CD8 cells that counter infection by certain HIV-1 strain infections (7) provided the critical identification of two chemokine receptor molecules, CXCR4 (formerly named LESTR/fusin) and CCR5 (formerly CXCR5), as cell surface coreceptors with CD4 for HIV-1 infection (8-13). Additional chemokine receptors CCR2 and CCR3 also

have been implicated as HIV-1 coreceptors on certain cell types (12-14). HIV-1-infected patients harbor predominantly macrophage-tropic HIV-1 isolates during early stages of infection, but accumulate increasing amounts of T cell-tropic strains just before accelerated T cell depletion and progression to AIDS. The identification of "dual"-tropic HIV-1 strains over the course of infection suggests that such strains may represent an intermediate between macrophage- and T cell-tropic populations (11-13, 15). This tropic transition indicates that viral adaptation from CCR5 to CXCR4 receptor use may be a key step in progression to AIDS (16).

EXHIBIT 4

Phylogenetic Relationships of Reverse Transcriptase and RNase H Sequences and Aspects of Genome Structure in the Gypsy Group of Retrotransposons¹

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The *gypsy* group of long-terminal-repeat retrotransposons contains elements having the same order of enzyme domains in the *pol* gene as do retroviruses. Elements in the *gypsy* group are now known from yeast, filamentous fungi, plants, insects, and echinoids. Reverse transcriptase and RNase H amino acid sequences from elements in the *gypsy* group—including the recently described *SURL* elements, *TED*, *Cfi1*, and *Ulysses*,—were aligned and analyzed by using parsimony and bootstrapping methods, with plant caulimoviruses and/or retroviruses as outgroups. Clades supported at the 95% level after bootstrapping include (1) 17.6 with 297 and (2) all of the *SURL* elements together. Other likely relationships supported at lower bootstrap confidence intervals include (1) *SURL* elements with *mag*, (2) 17.6 and 297 with *TED*, and this collective group with 412 and *gypsy*, (3) *Tf1* with *Cfi1*, (4) *IFG7* with *Del*, and (5) all of the retrotransposons in the *gypsy* group together, to the exclusion of *Ty3*. In contrast with an earlier analysis, our results place *mag* within the *gypsy* group rather than outside of a cluster that contains *gypsy* group retrotransposons and plant caulimoviruses. Several features of retrotransposon genomes provide further support for some of the aforementioned relationships. The union of *SURL* elements with *mag* is supported by the presence of two RNA binding sites in the nucleocapsid protein. Location of the tRNA primer binding site and the presence of a long open reading frame 3' to the *pol* gene support the 17.6-297-*TED*-412-*gypsy* cluster.

Introduction

Retrotransposons containing long terminal repeats (LTRs) have now been identified in the genomes of a number of organisms and can be divided into two groups on the basis of both phylogenetic analysis of amino acid sequences and structural features of the genome (Xiong and Eickbush 1988, 1990; Doolittle et al. 1989). In the *copia* group, with representatives from *Drosophila* (*copa* and 1731), yeast (*Ty1*), plants (*Tnt1*, *Tal-3*, *Ts1*, *Wis*, and *Bis*), and *Physarum* (*Tp1*), the integrase gene is located between the protease and reverse transcriptase genes. In the *gypsy* group, with representatives from insects (*gypsy*, 412, 17.6, 297, *mag*, *micropia*, and *Ulysses*), yeast (*Ty3* and *Tf1*), filamentous fungi (*Cfi1*), echinoids (*SURL* elements), and plants (*IFG7* and *Del*), the integrase gene is located 3' to the RNase H gene. The *gypsy* group of LTR retrotransposons is related to plant caulimoviruses and to retroviruses, on the basis of reverse transcriptase sequences (Xiong and Eickbush 1990).

1. Key words: retrotransposon, reverse transcriptase, RNase H.

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Here, we examine phylogenetic relationships among members of the *gypsy* group by using amino acid sequences from the reverse transcriptase and RNase H proteins. Previous phylogenetic analyses of the *gypsy* group include Doolittle et al. (1989) and Xiong and Eickbush (1990). Xiong and Eickbush (1990) included 10 elements from the *gypsy* group in their analysis of reverse transcriptase sequences. Since that time, sequences for *SURL* elements, *TED*, *Tf1*, *Cf1*, and *Ulysses* have become available. We also evaluate the distribution and evolution of structural features in these retrotransposons in the light of amino acid-based phylogenies. Several structural features corroborate phylogenetic analysis on the basis of amino acid sequences.

Methods

Amino acid sequences and features of retrotransposons were obtained from GenBank and from references given in figure 1. Sequences of representative plant caulimoviruses were also obtained from GenBank. Delineation of boundaries for the reverse transcriptase protein correspond to that used by Xiong and Eickbush (1990). Delineation of RNase H sequence boundaries roughly corresponds to the region identified by McClure (1991). Multiple alignments were made by using CLUSTAL (Higgins and Sharp 1988), and adjustments were made by eye when conserved residues defined in Xiong and Eickbush (1990) and McClure (1991) were not aligned. Maximum parsimony and bootstrapping were performed by using PAUP, version 3.0s (Swofford 1991), with gaps counted as missing data. Plant caulimoviruses and/or retroviruses were used as outgroups. Each step on a parsimony tree corresponds to a single amino acid replacement. Because exact methods of finding minimum-length trees could not be used for the complete set of sequences, a heuristic approach using 100 replications with random input orders was employed. We also used a starting tree consistent with the tree given in Xiong and Eickbush (1990) as a baseline for searching for shorter trees. A distance matrix based on the aligned amino acid sequences was constructed by using the Kimura (1983, p. 175) option of the PROTDIST program on PHYLIP and was analyzed by using the neighbor-joining method (Saitou and Nei 1987).

Results

Alignments

Figure 1 shows a multiple alignment of amino acid sequences from the reverse transcriptase region. Overall, this alignment is similar to that of Xiong and Eickbush (1990), and most of the conserved blocks in their alignment are retained in the present alignment. Figure 2 shows an alignment of sequences from the RNase H region.

Phylogenetic Trees

Two minimum-length trees containing 2,219 amino acid replacements were found for the combined reverse transcriptase/RNase H sequences. One of these trees, rooted by using plant caulimoviruses, is shown in figure 3. On the second tree (not shown) the *Tf1-Cf1* group and *IFG7-Del* groups switch positions, and *micropia* is closer to *Ulysses* than to the *SURL-mag* group. Also shown on the tree in figure 3 are the consensus results of 500 bootstrap replications. Results summarized in figure 3 show (1) a likely sister-group relationship (86%) of *TED* (from the cabbage looper *Trichoplusia ni*) with 17.6 plus 297 (from *Drosophila*), (2) a likely sister-group relationship (73%) between the plant retrotransposons *IFG7* and *Del*, (3) a likely sister-group relationship (80%) between *SURL* elements and *mag*, (4) a likely sister-group rela-

17.6	DFTKFTLTDRASDURLGAULSQDGH-----	PLSVISATLNHEHINYSTIKELLAIUWATKTFAYHLLOR
297	DFEKFULTTDRASDURLGAULSQDGH-----	PISEISATLNHEHINYSTIKELLAIUWATKTFAYHLLOR
TED	DTREFTULTDRASDURLGAULSQDGH-----	PUCYASATLNHEHINYSTIKELLAIUWATKTFAYHLLOR
412	DFSEKFCITDRASKQCORULQNHQHQHQL-----	PUNYASRAITKGESEKSTTEGELATIHAIHAIHFAPIVYQ
gypsy (Dm)	DFKQFULTDRASDURLGAULSQDGH-----	PTITISATLNHEHINYSTIKELLAIUWATKTFAYHLLOR
gypsy (Dv)	NFKQFPLTDRASDURLGAULSQDGH-----	PTITISATLNHEHINYSTIKELLAIUWATKTFAYHLLOR
SURL (Dm)	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
SURL (Sp)	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
SURL (Tg)	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
SURL (Lv)	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
mag	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
micropia	DCCKLTKLSARASQKQIGIURLVQQDQDIU-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
Ulysses	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
IF07	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
De1	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
Tf1	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
Cf11	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
Ty3	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
COYVU	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
CERU	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
FIGWORT	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
CRU	DFRPFPTIQCDASHVGLVQLFQDDEQER-----	PIYASASHTDRETRYAQIKELLAIUYACERFHQVIVQ
17.6	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
297	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
TED	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
412	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
gypsy (Dm)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
gypsy (Dv)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
SURL (Dm)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
SURL (Sp)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
SURL (Tg)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
SURL (Lv)	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
mag	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
micropia	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
Ulysses	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
IF07	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
De1	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
Tf1	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
Cf11	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
Ty3	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
COYVU	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
CERU	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
FIGWORT	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----
CRU	-----DPHS-KLTRH-----	-----RVKLSSEFDI-----

FIG. 2.—Alignment of amino acid sequences from the RNase H region for the *gypsy* group of retrotransposons and several plant caulimoviruses. Abbreviations are given in fig. 1. An asterisk (*) denotes a stop codon.

tionship (81%) between *Tf1* (from fission yeast) and *Cf11* (from the fungal tomato pathogen *Cladosporium fulvum*), and (5) a possible clade (62%) containing 17.6, 297, *TED*, 412, and *gypsy*. In addition, *Ty3* is an outgroup to all other retrotransposons in the *gypsy* group on 70% of the bootstrap trees. *Ulysses* and *micropia* group with *SURL* elements and *mag* on both minimum-length trees, but this association does not hold up after bootstrapping. Likewise, the minimum-length tree shown in figure 3 supports a clade containing all of the retrotransposons that occur in metazoans, but this branch does not occur on the second minimum-length tree, nor is it supported by bootstrapping. In contrast to the tree in figure 3, the shortest tree consistent with that of Xiong and Eickbush (1990) is 30 steps longer, at 2,249 steps.

When we converted our sequence alignments to distances by using the Kimura option of PROTDIST (PHYLIB, version 3.5; Felsenstein 1993) and then employed the neighbor-joining method, the resulting tree (not shown) showed some differences from the minimum-length trees, but all of the branches that are supported at the 50% level in figure 3 are also supported on the neighbor-joining tree.

Minimum-length trees (not shown) based on reverse transcriptase versus RNase H sequences exhibit several conflicts; for example, *SURL* elements cluster with *mag* on reverse transcriptase trees but cluster with the two *gypsy* elements on RNase H trees. However, all of the conflicts involve branches that are not supported after boots-

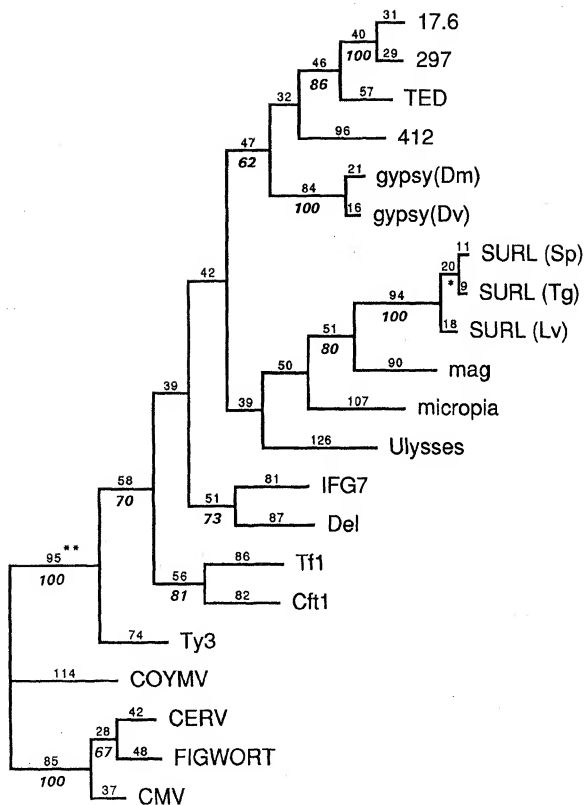


FIG. 3.—One of two minimum-length trees at 2,219 replacements. Numbers above the line are the number of amino acid replacements, and numbers below the line are the percentages, from 500 bootstrap trials, that support the clade. One asterisk (*) denotes a value of 99%, and two asterisks (**) denote the branch of this tree that the root would be on if the reverse transcriptases of the seven retroviruses were used for rooting.

trapping. Bootstrapping the reverse transcriptases and RNase H sequences, respectively, provides support for the following: 17.6 plus 297 (94% and 97%), and for this group with *TED* (85% and 64%); the two *gypsy* elements together (100% and 100%); the three *SURL* elements together (100% and 100%) with *SURL* (Sp) and *SURL* (Tg) as nearest neighbors (96% and 90%); and *Tf1* plus *Cf11* (60% and 73%). In addition, bootstrapping reverse transcriptase sequences provides support for *SURL* elements with *mag* (60%), *IFG7* plus *Del* (71%), and all of the retrotransposons together, except *Ty3* (52%).

Features of *gypsy*-like Elements

Table 1 summarizes the distribution of seven different features of retrotransposons in the *gypsy* group. The phylogenetic significance of these features is discussed below.

Discussion

Xiong and Eickbush (1988, 1990) previously examined relationships among retrotransposon elements, including retrotransposons in the *gypsy* group, on the basis of reverse transcriptase sequences. One of the differences on the Xiong and Eickbush (1990) tree is that *mag* is outside of a cluster containing other retrotransposons in the *gypsy* group as well as plant caulimoviruses. To test this hypothesis with our data, it was necessary to include retroviruses as an outgroup to the collective group. We limited this analysis to reverse transcriptase sequences because of the difficulty in aligning RNase H sequences. Retroviruses clearly root the tree (not shown) such that the plant-caulimovirus and retrotransposon groups (including *mag*) are each monophyletic.

Two other differences on the Xiong and Eickbush (1990) tree are as follows: (1) *Ty3* is not peripheral to other *gypsy* retrotransposons but occupies a position close to *IFG7* and *Del*, and (2) 412 is the most peripheral member of the *gypsy* cluster, except *mag*. Whether we (1) use parsimony or neighbor-joining methods, (2) include RNase H and reverse transcriptase or just reverse transcriptase sequences, or (3) restrict our analysis to the reverse transcriptase sequences available to Xiong and Eickbush (1990), *Ty3* occupies the most peripheral position among retrotransposons in the *gypsy* group, and 412 clusters with the insect elements *gypsy*, 297, 17.6, and *TED*.

The overall congruence between reverse transcriptase and RNase H bootstrap trees indicates that a similar phylogenetic signal is present in both, although, when taken separately, each of these proteins provides less resolution than they do in combination with each other. One of the implications of the overall congruence between bootstrap trees is that reverse transcriptase and RNase H have similar evolutionary histories without any interelement recombination that might cause striking differences.

If *Ty3* is taken as an outgroup to all of the other retrotransposons, then the implied primitive character states for the characters in table 1 are +1 ribosomal frameshifting, one RNA binding site, tRNA methionine, a +2 location of the tRNA primer binding site (PBS), and lack of a long open reading frame (ORF) 3' to the *pol* gene. On the basis of these designations of primitive character states, several of the aspects of genome structure given in table 1 offer additional support for some of the branches on the tree in figure 3. First, 17.6, 297, and *TED* are united by the putative shared derived character of tRNA serine, although a putative tRNA serine also occurs in *Cf11* (McHale et al. 1992). Second, 17.6, 297, *TED*, 412, and *gypsy* share a number of putative derived characters, including a long ORF 3' to the *pol* gene, a 1-bp overlap of the 5' LTR and the tRNA PBS, and -1 frameshifting of the *pol* gene relative to the *gag* gene, as well as the absence of RNA binding sites in the nucleocapsid protein. While two of these derived characters have evolved elsewhere on the tree (i.e., -1

Table 1
Select Features of Retrotransposons Related to the *gypsy* Element of *Drosophila*

FEATURE	RETROTRANSPOSON ^a													
	17.6	207	TED	412	<i>gypsy</i> ^b	SURL ^c	Mag	<i>microplia</i>	Ulysses	IFG7	Del	ITI	CHI	Ty3
1. Ribosomal Frameshifting	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No	No	No	Yes	Yes
No. of nucleotides ^d	-1	-1	-1	-1	-1	0	-1	+	+	No	No	No	-1	+1
2. No. of RNA binding sites	0	0	0	0	0	2	2	2	0	1	1	0 (?)	1	1
3. tRNA PBS	Serine	Serine	Serine	Arginine	Lysine	Methionine	Arginine	Leucine	Lysine	Methionine	Methionine	?	Serine (?)	Methionine
4. Location of tRNA PBS ^e	-1	-1	-1	-1	-1	0	+1	0	+3	+2	+3	?	0	+2
5. Envelope (?) ORF ^f	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No
6. LTR length (in bp)	512	415	273	481-571	479-482	254	77	474-504	2,136	333	2,406-2,415	358	427	340
7. Element length (in bp)	7,439	6,995	7,510	7,400	7,469	5,266	4,564	5,500	10,653	5,944	9,345	4,941	6,968	5,428

^a Boxes enclose clades of fig. 3.

^b Dm strain.

^c Tg strain.

^d No. of nucleotides that must "read" to change from the frame reading "xxx" to that reading "yyy."

^e Relative to the 5' LTR. Minus (-) values are base pair of overlap; plus (+) values are the distance between.

^f A number of retrotransposons contain long (~1,500-bp) ORFs located between the *pal* gene and the 3' LTR. In retroviruses, the ORF in this region codes for proteins that are essential in the extracellular stage of the life cycle; however, there is as yet no direct evidence that the similarly placed ORF that occurs in some retrotransposons codes for homologous proteins.

frameshifting also occurs in *Cf11* and *mag*, and RNA binding sites are absent in *Ulysses*), the presence of a long ORF 3' to *pol* and a -1 location of the tRNA PBS are unique to this subset of the *gypsy* group. Third, the putative relationship between *mag*, *SURL* elements, and possibly *micropia* is potentially strengthened by the exclusive occurrence of two RNA binding sites in the nucleocapsid protein in all of these elements. Most retroviruses also possess two RNA binding sites, but in the somewhat more closely related plant caulimoviruses there is only a single site. Further support for the alliance between *mag* and *SURL* elements comes from the observation that the number of amino acids separating the two RNA binding sites is identical in these elements. *Micropia*, in turn, has 14 additional amino acids that separate the first and second RNA binding sites. The plant elements *Del* and *IFG7* share a number of features, such as a single RNA binding site, a single ORF containing the *gag* and *pol* genes, and a tRNA methionine PBS, but these features appear primitive on the basis of their occurrence in *Ty3*.

The long LTRs in *Ulysses* and *Del* appear homoplastic on the basis of other evidence discussed above, whereas the short LTRs in *mag* are unique to this element. Element length ranges from 4,564 bp in *mag* to 10,653 bp in *Ulysses* and reflects the differences in LTR length. Among other elements, most of the variation results not from differences in LTR length but rather from the additional ORF 3' to the *pol* gene.

It is interesting that, for the tree in figure 3, all of the animal retrotransposons occur on one branch, whereas the two plant elements occur on a second branch. The separate clusters of plant and animal retrotransposons suggest that the host phylogeny imposes a distinct signature on the phylogeny of the retrotransposons; Flavell (1992) previously noted predominantly plant and animal groups for the *copa* group of retrotransposons as well. Flavell (1992) has also characterized the *copa* group as lacking ribosomal frameshifting, whereas in the *gypsy* group the *gag* and *pol* genes are always overlapping. However, the presence or absence of overlapping *gag* and *pol* genes is shown here to exhibit more variation in the *gypsy* group than was previously recognized.

In conclusion, our understanding of the phylogeny of the *gypsy* group of retrotransposons is enhanced by considering not only amino acid sequences but also genetic features of these elements. Some features (e.g., long 3' ORF) show little or no homoplasy, whereas others (e.g., type of tRNA PBS) are labile and show much more homoplasy.

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EXHIBIT 5



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Reverse Transcriptase Motifs in the Catalytic Subunit of Telomerase

Joachim Lingner,* Timothy R. Hughes, Andrej Shevchenko, Matthias Mann,† Victoria Lundblad,† Thomas R. Cech†

Telomerase is a ribonucleoprotein enzyme essential for the replication of chromosome termini in most eukaryotes. Telomerase RNA components have been identified from many organisms, but no protein component has been demonstrated to catalyze telomeric DNA extension. Telomerase was purified from *Euplotes aediculatus*, a ciliated protozoan, and one of its proteins was partially sequenced by nanoelectrospray tandem mass spectrometry. Cloning and sequence analysis of the corresponding gene revealed that this 123-kilodalton protein (p123) contains reverse transcriptase motifs. A yeast (*Saccharomyces cerevisiae*) homolog was found and subsequently identified as EST2 (ever shorter telomeres), deletion of which had independently been shown to produce telomere defects. Introduction of single amino acid substitutions within the reverse transcriptase motifs of Est2 protein led to telomere shortening and senescence in yeast, indicating that these motifs are important for catalysis of telomere elongation *in vivo*. *In vitro* telomeric DNA extension occurred with extracts from wild-type yeast but not from est2 mutants or mutants deficient in telomerase RNA. Thus, the reverse transcriptase protein fold, previously known to be involved in retroviral replication and retrotransposition, is essential for normal chromosome telomere replication in diverse eukaryotes.

Replication of chromosome ends, or telomeres, requires specialized factors that are not essential for replication of internal chromosome sequences. Conventional DNA polymerases cannot fully replicate blunt-ended DNA molecules (1) or eukaryotic chromosomes (2), which contain 3'-terminal extensions. The key to end replication is telomerase, a ribonucleoprotein (RNP) enzyme that synthesizes the telomeric DNA repeats (3). The template for telomeric repeat synthesis is provided by the RNA subunit, which has been identified, cloned, and sequenced in ciliated protozoa (4, 5), yeast (6, 7), and mammals (8).

A telomerase RNP was first purified from *Tetrahymena* (9). Two protein components, p80 and p95, were specifically associated with the RNA subunit. Human, mouse, and rat homologs of *Tetrahymena* p80 have since been identified and found to be associated with telomerase (10). Although this evolutionary conservation suggests that p80 and p95 have important roles

in telomere replication, their specific functions remain unclear. Neither protein has been reported to be essential for telomere synthesis, and neither has significant simi-

larity to known polymerases or reverse transcriptases (11).

Telomerase RNP has also been purified from *Euplotes aediculatus*, a hypotrichous ciliate only distantly related to *Tetrahymena* (12). The hypotrichs present a special opportunity for telomere studies because their macronuclei contain millions of gene-sized DNA molecules. Each cell has about 8×10^7 telomeres (13) and about 3×10^7 molecules of telomerase (12). Measurements of the specific activity of telomerase throughout the purification indicated that the major activity present in macronuclear extracts was purified (12). The active telomerase complex had a molecular mass of ~230 kDa, corresponding to a 66-kDa RNA subunit and two proteins of 123 kDa and ~43 kDa (12). Photocross-linking experiments implicated the larger protein in specific binding of the telomeric DNA substrate (14).

Here we characterize the p123 component of *Euplotes* telomerase and show that it contains sequence hallmarks of reverse transcriptases. Furthermore, it is the homolog of a yeast protein, Est2p, shown previously to function in telomere maintenance. Our genetic and biochemical analyses show that the reverse transcriptase motifs of Est2p are essential for telomeric DNA synthesis *in vivo* and *in vitro*. We propose that telomerase, frequently called "a specialized reverse transcriptase," is in fact a reverse tran-

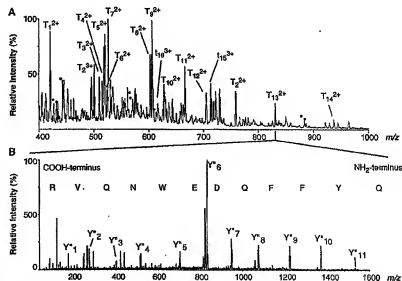


Fig. 1. Sequencing of the p123 subunit of telomerase by nanoelectrospray tandem mass spectrometry. (A) Mass spectrum of the unseparated peptide mixture. All peptides that were sequenced completely or partially are marked by the letter T or t, respectively (15). The eight peptide ions from which sequence tags were generated are marked by filled circles. Most unlabeled peaks correspond to trypsin autolysis products. (B) Tandem mass spectrum of the doubly charged precursor at the mass-to-charge ratio (m/z) of 830.4 in (A). Interpretation of the fragment ion mass in (B) and comparison with the esterified form of the peptide allowed the sequence assignment.

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Two of the peptide sequences were used to design degenerate polymerase chain reaction (PCR) primers (arrows in Fig. 2) to amplify a portion of the macronuclear gene encoding p123. A genomic library was prepared from macronuclear DNA and screened with this fragment to isolate the full-length gene (19). The p123 gene was found to be encoded by a 3279-base pair (bp) macronuclear chromosome containing an uninterrupted 1031-amino acid open reading frame. In a Southern (DNA) blot experiment the PCR fragment hybrid-

ized to a single macronuclear chromosome of ~ 3.3 kb (20). The open reading frame predicts a protein of 122,562 daltons, corresponding to the size estimated by SDS-polyacrylamide gel electrophoresis of purified protein [120 kD (12)]. More than 150 amino acids identified in the purified polypeptide by mass spectrometry could be assigned in the open reading frame (Fig. 2). This includes all 14 peptides that were completely sequenced. The tandem mass spectra of 10 additional peptides also

matched the gene sequence through partial sequences or peptide sequence tags (21).

Reverse transcriptase motifs in *Euplotes* p123 and its yeast homolog Est2p. In a BLAST search of protein databases, *Euplotes* p123 was found to be most similar to *Saccharomyces cerevisiae* Est2p ($P = 7 \times 10^{-7}$) and to a group II intron-encoded reverse transcriptase from the cyanobacterium *Calothrix* ($P = 2 \times 10^{-4}$) (22, 23). Yeast Est2p has a predicted molecular

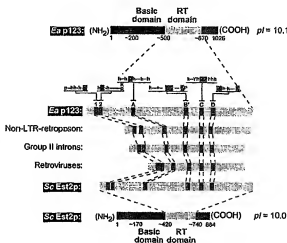


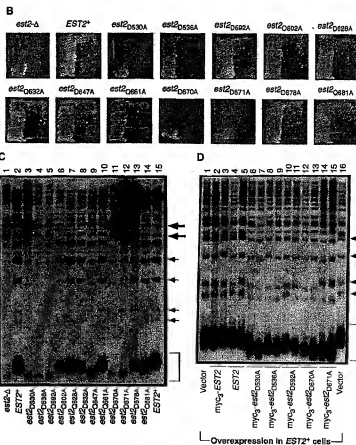
Fig. 3. Block diagrams of p123 and Est2p and comparison of the reverse transcriptase (RT) domains with those of other reverse transcriptases. The spacing of sequence motifs (red) is diagnostic for each reverse transcriptase family (27). In the consensus sequence, abbreviations are as in Fig. 2. The isoelectric point (*pI*) is the pH at which the protein has no net charge.



Fig. 2. Sequence alignment of *Euploies* (Ea) p123 and yeast [*S. cerevisiae* (Sc)] Est2p (50). Identical amino acids are noted in boldface. The PCR primers used to amplify a portion of the gene are indicated by the arrows. Assigned reverse transcriptase motifs [designated by letters (26) or alternatively by numbers in parentheses (27)] are shown in orange, with the most highly conserved amino acids in red. In the consensus sequences of the motifs, h designates a hydrophobic amino acid, p a polar amino acid, and + a positively charged amino acid.

acid. The underlined sequences in p123 are the 14 peptides completely sequenced by nanoelectrospray tandem mass spectrometry. The dashed lines below the p123 sequence indicate another 10 peptides whose tandem mass spectra matched the sequence. One of the peptides contained an acetylated methionine (solid triangle) at its NH₂-terminus, indicating that it was the NH₂-terminal peptide of the protein. The nucleotide sequence of the *E. coli* p123 gene has been deposited in GenBank (accession number U95864).

the same pathway as *TLC1*, the gene encoding the telomerase RNA subunit (6), suggesting that the EST genes encode either components of the telomerase or positive regulators of its activity. The homology of yeast *Est2p* with *Euplotes* p123, the latter isolated because of its physical asso-



ciation with telomerase RNA and its copurification with telomerase activity, supported the proposal that both proteins are intrinsic subunits of their respective telomerases.

Exopolys p123 contains reverse transcriptase motifs, and the alignment reveals the presence of these motifs in a similar region of *Est2p* (Fig. 3). The primary sequences of reverse transcriptases are highly divergent: Only a few amino acids are absolutely conserved within separate short motifs (26, 27), but these motifs are believed to form a common tertiary fold. Both p123 and *Est2p* contain these key conserved amino acids, most notably the three invariant aspartates in motifs A and C, which are thought to be directly involved in catalysis (Fig. 2). Conserved motifs are spaced differently in the two major branches of reverse transcriptases, those encoded by retroviruses and long terminal repeat (LTR) retrotransposons and those encoded by non-LTR retrotransposons and group II introns (27). The spacing of sequence motifs in p123 and *Est2p* resembles that in the latter branch. However, the interval between motifs A and B' in p123 and *Est2p* is unusually large (Fig. 3), suggesting that these two polypeptides may be members of a previously unknown subcategory.

Requirement of the reverse transcriptase motifs for *Est2p* function in vivo. The presence of reverse transcriptase motifs in both p123 and *Est2p* suggests that this region

may define the catalytic active site of telomerase. To test the importance of these motifs for *Est2p* function, we used site-directed mutagenesis to change conserved and non-conserved aspartic acid (D) and glutamine (Q) residues in and around motifs A, B', and C to alanine (A) (Fig. 4A). Each mutant, present on a single-copy *ARS CEN* plasmid, was tested for in vivo function in a complementation assay. Plasmids were transformed into the *est2-Δ* strain (Δ designates deletion), in parallel with either the empty vector or an *EST2+* plasmid. Transformants were assessed for the senescence phenotype (Fig. 4B) and for chromosome telomere length (Fig. 4C).

Consistent with the prediction that the reverse transcriptase motifs are required for *Est2p* function, mutation of any of the three conserved aspartates in motifs A and C prevented normal telomerase activity. Transformants expressing these mutant proteins became senescent and had shortened telomeric tracts, phenotypes indistinguishable from those of the null mutant (Fig. 4, B and C). Furthermore, a bypass pathway for telomere maintenance (28) was evident in these three mutant strains. Activation of this alternative pathway occurs as the result of a global amplification and rearrangement of both telomeric G-rich repeats and subtelomeric regions, and has only been observed in *ex1* and *dcl1* mutant strains with a severe telomere shortening phenotype (24, 29). A feature of this pathway is the amplification

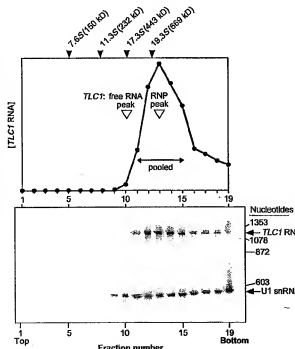
of two subtelomeric bands (Fig. 4C); these diagnostic restriction fragments were substantially amplified only in the *est2* null mutant and the three proposed active site mutants.

Mutations of amino acids other than the three most conserved aspartates had less severe or no phenotypic effects. The residue *Asp*⁵¹⁶ of motif A is conserved between *Est2p* and p123, and the D536A mutation (*Asp* mutated to Ala at position 536) caused substantial telomere shortening and a modest senescence phenotype. Of the conserved residues tested, *Gln*⁶¹² of motif B' was the only one that was functionally insensitive to replacement with alanine. However, this glutamine is not strictly conserved in reverse transcriptases (27), and when it is changed to alanine in human immunodeficiency virus-1 (HIV-1) reverse transcriptase, polymerase activity in vitro is reduced but not completely eliminated (30). In contrast to the phenotypes seen upon mutation of the semiconserved amino acids, mutation of six of the seven nonconserved amino acids tested showed little or no alteration of *Est2p* function.

Two observations indicate that stable *Est2* protein was produced in the five *est* mutants with a diminished capacity to complement the *est2-Δ* strain. First, Myc⁺ epitope-tagged versions of each mutant protein were visualized immunologically after immunoprecipitation (31). Second, overexpression of each of the five mutant alleles in a wild-type yeast strain with a functional chromosomal *EST2+* gene resulted in telomere shortening (Fig. 4D), whereas overexpression of the wild-type *EST2* gene had little effect. The dominant-negative phenotype shows that each mutant protein is being made and suggests that excess mutant *Est2p* can titrate components away from the wild-type telomerase complex.

Requirement of *Est2p* for telomerase activity in vitro. If *Est2p* is the catalytic protein subunit of telomerase, then telomerase activity should be abolished in *est2* mutant extracts. An in vitro assay was developed with extracts fractionated by glycerol gradient centrifugation (32). Telomerase-containing fractions were identified by detection of the RNA subunit on Northern blots (Fig. 5). Yeast telomerase sedimented as a 19S to 20S particle, substantially faster than the sedimentation of the deproteinized telomerase RNA (~17S). Telomerase-containing glycerol gradient fractions were pooled, concentrated, and tested for the ability to elongate a single-stranded telomeric oligonucleotide (Fig. 6). An activity was detected in wild-type extracts that had the characteristics of telomerase. It was dependent on the presence of oligonucleotide substrate and fractionated extract (Fig. 6A,

Fig. 5. Sedimentation of telomerase. Yeast extract was fractionated on a glycerol gradient (32), and telomerase RNA was detected by Northern blotting (bottom) and its concentration quantified on a PhosphorImager (top). Detection of U1 snRNP served as an internal control. Fractions pooled for activity assays are indicated. Telomerase RNP sedimented as a 19S to 20S particle, whereas deproteinized telomerase RNA sedimented at ~17S. The sedimentation value was determined relative to marker proteins that were run in parallel gradients and that had sedimentation coefficients of 7.6S (alcohol dehydrogenase), 11.3S (catalase), 17.3S (apoferritin), and 19.3S (thyroglobulin).



lanes 1 to 3). Addition of T and G residues occurred in an ordered manner consistent with the expected alignment of substrate and RNA template (Fig. 6A, lanes 5 and 6). The activity was sensitive to low concentrations of ribonuclease (RNase A) and was not stimulated by adenosine triphosphate (ATP) (Fig. 6B). These characteristics, in addition to the observed single round of extension of primer (Fig. 6A), are similar to those of the telomerase activity described by Blackburn and co-workers (33). A different activity described as telomerase by Lue and Wang (34) gives rise to long products and is stimulated by ATP. This latter activity was not detectable in our telomerase-containing glycerol gradient fractions.

A telomerase RNA template mutation that alters the specificity of nucleotide incorporation to produce a *Hae* III restriction site (6) provides an additional test for the authenticity of the *in vitro* telomerase assay. An extract of this *TLCl-1* (*Hae*III) mutant, fractionated on a glycerol gradient, gave the predicted extension of the telomeric oligonucleotide only in the presence of deoxycytidine triphosphate (dCTP) (Fig. 6C, lanes 6 to 8), a nucleotide that has no effect on extension by a wild-type extract (Fig. 6C, lanes 2 and 3). This nucleotide specificity change supports the dependence of the assay on the *TLCl* RNA. Because the *TLCl-1* (*Hae*III) strain also undergoes senescence (29), this result also provides confidence that telomerase activity can still be detected in senescing cells, as long as they are not subcultured too extensively.

We then assayed fractionated extracts from *est2-Δ* and *tcl1-Δ* strains for telomerase activity (Fig. 6D). As expected, no activity was detectable in *tcl1-Δ* yeast, which has the gene for telomerase RNA deleted. In the *est2-Δ* strain, telomerase RNA was still assembled into an RNP, as assessed by glycerol gradient centrifugation and Northern blotting (32), but telomerase activity was completely absent. This indicates that *Est2p* is essential for telomerase activity. As described above, the absence of activity is not simply a secondary consequence of senescence. We also measured telomerase activity in extracts from *est2-Δ* and strains expressing two of the proposed active site mutants in the presence of the chain-terminating analog dGTP (Fig. 6E). According to the proposed primer-template alignment, extension should terminate after addition of two nucleotides. A practical advantage is the higher signal-to-noise ratio obtained when all products are concentrated in one or two bands. Again, activity was dependent on functional *TLCl* and *EST2* genes.

Telomerase structure. The presence of a reverse transcriptase domain in the catalytic subunit of telomerase provides a framework

for exploring the structure and mechanism of this enzyme. Reverse transcriptases have been studied in great detail, and the three-dimensional structure of HIV-1 reverse transcriptase has been solved (35). The structure can be compared with a right hand with fingers, palm, and thumb, with the active site residing in the palm (36). A model for telomerase structure based on that of HIV-1 reverse transcriptase (HIV-1 RT) is shown in Fig. 7 with the telomerase RNA and a telomeric DNA substrate superimposed.

The catalytic subunit of telomerase has several features that distinguish it from other reverse transcriptases. Telomerase uses only a small portion of its RNA subunit as a template. The borders of this template

must somehow be recognized. Furthermore, during processive synthesis of telomeric repeats the substrate translocates from one end of the template to the other by an as yet unknown mechanism. The large gap between motifs A and B' of telomerase p123 and *Est2p* indicates an unusual finger domain structure. In HIV-1 RT this domain may be involved in template strand binding (35, 36); whether and how it contributes to the unusual reaction mechanism of the telomerase RNP remain to be investigated. Finally, the telomerase protein is stably associated with its RNA subunit, as shown by our isolation of the *Explotes* p123-RNA complex and by immunoprecipitation of the yeast RNA subunit with *Est2p* (31).

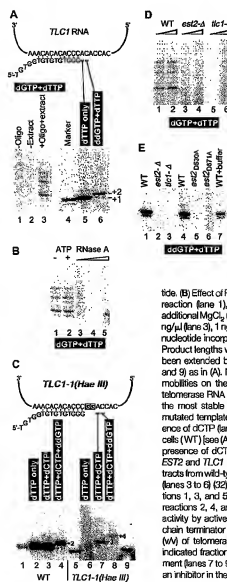


Fig. 6. *In vitro* functional analysis of reverse transcriptase motifs in *Est2p*. Telomerase was partially purified by glycerol gradient centrifugation and assayed for the ability to extend a telomeric DNA substrate (32). In the assay [α^{32} P]dGTP was included to visualize products elongated by 1, 2, 3, or nucleotides (+1, +2, +3, or +4). (A) The telomerase RNA template region maximally base-paired to the DNA substrate is indicated schematically. Product lengths were determined relative to the same DNA substrate extended by one nucleotide at the 3' end by reaction with [α^{32} P]dGTP and terminal deoxynucleotidyl transferase (lane 4). Up to seven nucleotides were added in the presence of dGTP and dCTP (lane 3), one nucleotide in the presence of only dCTP (lane 5), and two nucleotides in the presence of dCTP and the chain-terminating analog dGTP (lane 6). (B) Effect of RNase A and ATP on telomerase activity. Standard reaction (lane 1), standard reaction plus 1 mM ATP and 1 mM additional $MgCl_2$ (lane 2), and standard reaction plus RNase A at 0.1 ng/ μ l (lane 3), 1 ng/ μ l (lane 4), and 10 ng/ μ l (lane 5). (C) Specificity of nucleotide incorporation dictated by the RNA template sequence. Product lengths were determined relative to DNA markers that had been extended by [α^{32} P]dGTP (lane 1) or [α^{32} P]dCTP (lanes 5 and 9) as in (A). Note that these two markers had slightly different mobilities on the polyacrylamide gel. The mutant *TLCl-1* (*Hae*III) telomerase RNA template is indicated with the substrate bound in the most stable register. Consistent with this alignment and the mutated template sequence, efficient extension required the presence of dCTP (lanes 6 and 7). Telomerase in extract from *TLCl*-wt cells (WT) (lane 8) for template sequence) was not influenced by the presence of dGTP (lanes 2 and 3). (D) Requirement of functional *EST2* and *TLCl* products for telomerase activity. Fractionated extracts from wild-type (lanes 1 and 2) and the indicated mutant strains (lanes 3 to 6) (32) were tested at two extract concentrations. Reactions 1, 3, and 5 contained 10% (w/v) of telomerase fraction, and reactions 2, 4, and 6 contained 20%. (E) Allelation of telomerase activity by active site mutations in *Est2p*. All assays included the chain terminator dGTP (100 μ M). The reactions contained 10% (w/v) of telomerase fraction (lanes 1 to 6) or 5% of each of the indicated fractions (lanes 7 to 9). The results of the missing experiment (lanes 7 to 9) indicate that the absence of activity is not due to an inhibitor in the mutant extracts.

This last feature distinguishes telomerase from the retroviral and LTR retrotransposon reverse transcriptases, but is similar to some mitochondrial and group II intron-encoded reverse transcriptases that also form complexes with their RNA templates (37).

Reverse transcriptase essential for chromosome replication in diverse eukaryotes. Reverse transcriptases have not previously been considered essential for normal cell physiology. Initially discovered as retroviral enzymes that catalyze the defining RNA-to-DNA step of retroviral replication (38), they were later found to mediate the transposition of DNA elements within eukaryotic genomes through an RNA intermediate (39). Reverse transcriptases are also present in some prokaryotes (40) and in *Neospora mitochondria* (41), where they replicate genetic elements that are nonessential to their "host." Our discovery that a structurally related enzyme is essential for chromosome replication and cell division provides another example of the opportunism of nature: once a useful protein motif is stumbled upon, natural selection promotes its exploitation in diverse ways.

The evolutionary relationship between telomerase and the other reverse transcriptases is intriguing. It is well established that retroviruses acquired oncogenes such as *v-src*, *v-abl*, *v-ras*, and *v-fos* from cellular genomes. According to Temin's provirus hypothesis, retroviruses also acquired their reverse transcriptase gene from normal cells, where the enzyme presumably contributed to some normal cellular process (42). Could this cellular source have been the telomerase p123/EST2 gene, which mutated so that the protein product used an

exogenous rather than an intrinsic RNA template? Alternatively, telomerase and the reverse transcriptases encoded by retrotransposons and retroviruses may all be descendants of an ancestral protein that emerged from an "RNA world" (43).

Telomere replication in the fruit fly *Drosophila* has been mysterious because this organism does not have short repeated telomeric sequences and presumably no telomerase. Rather, the non-LTR retrotransposons *HeT-A* and *TART* cap the chromosome ends (44). The *TART* reverse transcriptase is closely related to p123 and Est2p, which suggests that the *Drosophila* telomere replication machinery may in fact not be so different from that of other eukaryotes (45).

We have no satisfactory explanation for the lack of correspondence between the *Euplotes* and yeast p123/Est2p proteins and the *Tetrahymena* p80 or p95 protein (9). The small protein subunit of *Euplotes* telomerase (p43) also shows no similarity to the *Tetrahymena* proteins (46), and the complete yeast genome sequence does not reveal obvious p80 and p95 homologs. There are three possible explanations: (i) *Tetrahymena* may have a different telomerase in which p80 and p95 provide the active site (that is, telomerase was invented more than once in evolution). (ii) *Tetrahymena* may have two telomerases, one containing p80 and p95 and one (unisolated) containing a p123/Est2p homolog (for example, one telomerase for de novo telomere formation during macronuclear development and one for telomere replication). (iii) The *Tetrahymena* p80-p95-RNA complex may not be an active enzyme but may require a p123/Est2p subunit that was underrepresented upon purification of the particle.

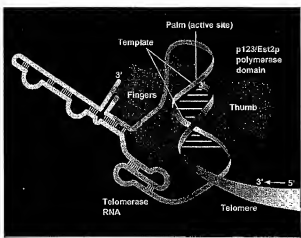
Mass spectrometric methods have recently become very successful for the identification of proteins whose genes are already partially or completely contained in sequence databases (47). The sequencing of more than 150 amino acids of the p123 telomerase subunit at protein amounts too low for chemical methods shows that mass spectrometry is now also valuable for sequencing previously unidentified proteins.

Telomerase activation accompanies the immortalization of cultured mammalian cells and is also a common property of human tumor cells (48). Thus, telomerase is considered to be a potential target for the development of tumor-specific drugs. Certain reverse transcriptase inhibitors developed as anti-HIV drugs have already been tested against telomerase with some success (49). The finding that the telomerase active site is related to that of known reverse transcriptases is expected to stimulate such efforts.

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Fig. 7. Model of telomerase as an RNA-reverse transcriptase complex. The p123/Est2p subunit (green) is based on the right hand model of HIV-1 RT (35); thumb and fingers extend toward the nascent RNA. Motifs A, B', C, and D are in the palm, and the active site aspartates are near the 3' end of the telomeric DNA substrate (red). The RNA subunit (purple) has its template region in the palm; the location of the remainder of the RNA is unknown and is shown schematically in its secondary structure representation (5). Additional protein subunits may be associated (not shown). The telomeric DNA substrate is shown base-paired but not intertwined with the RNA subunit. The extent of base pairing and the sites of interaction of the nucleic acids with the protein are not known.



- dem mass spectra were acquired with 0.2-delta resolution. The mass resolution was sufficient to assign unambiguously an amino acid series to the identified COOH-terminal ion series. The procedure was repeated with the esterified peptide mixture as described (16). The following peptide sequences (59) were assigned: T1, VLLFGK; T2, DYNEDGQVLYKAR; T3, ILKNGVAK; T4, SFLLNNLTTHYR; T5, TLYSLWLR; T6, ELTAEEVQK; T7, AMLNELSR; T8, LQTSPLSPSK; T9, (A)GTLFTNLTR; T10, (L)TALPMNLNLR; T11, LPAQTLVATPR; T12, LSEWAGVQTSK; T13, QYFQDQWNGVR; and T14, (L)DSMNPFNNNNLMLR. The partial sequence EVDVD was contained in the NH₂-terminal peptide 115 (EVDVDVNDGADGIRNAK) and the sequence LFAT in the peptide 116 (LFATMDIEK) and the two discrepancies between the sequence determined by mass spectrometry and that deduced from the DNA sequence. The glutamine in peptide T2 was a lysine, and the order of the first two amino acids in T12 was reversed. In the above sequences, L signifies leucine or isoleucine, two isobaric amino acids that cannot be distinguished by our methods, and the parentheses indicate that the order of the first two amino acids could not be distinguished.
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 31. Est2p was overexpressed under control of the ADH promoter as a Myc-tagged fully functional deactivator. Extracts were immunoprecipitated with an antibody to c-Myc. Wild-type and mutant Est2 proteins were precipitated with the antibody and detected on an immunoblot. Telomerase RNA was communally precipitated with Est2p only in strains harboring the Myc-tagged gene and was detected on a Northern RNA blot. U1 snRNA was not substantially precipitated but was detected in the crude extract.
 32. Yeast was grown in yeast extract, peptone, and dextrose (YPD) medium to an optical density (600 nm) of 1.8, and extracts were prepared as described (A. Ascoli and B. Scherer, *EMBO J.* 14, 4001 (1995)) with the following modifications: Cell pellets were resuspended in two volumes of extract buffer [20 mM Tris-acetate (pH 7.5), 300 mM potassium glutamate, 1 mM MgCl₂, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol (DTT)], and 0.5 mM phenylmethylsulfonyl fluoride. Extracts were concentrated threefold on centricon 30 (Amicon) and fractionated by glycerol gradient centrifugation in extract buffer containing 15 to 40% glycerol (12). Centrifugation was performed in a SW55Ti rotor (Beckman) at 304,000g_{max} for 12 hours at 4°C. Nucleon fractions were collected from the top of the gradient and assayed for the telomerase RNA subunit TLC1p by Northern blotting. In est2-Δ and est2 mutant strains, telomerase did not show a major shift in its sedimentation coefficient relative to the wild type, but the large size of the RNA and the limited resolution of the glycerol gradients (Fig. 8) would have prevented a small shift from being observed. Telomerase-containing fractions were pooled and concentrated fivefold on centricon 30. Telomerase assays (10 μl) contained 20 mM Tris-acetate (pH 7.5), 1 mM MgCl₂, 30 mM potassium glutamate, 1 mM DTT, 1 μM diphosphonate substrate [5'-TGTTGGTGGTGGG-3'], 100 μM deoxy- or dideoxyguanosine triphosphate (dGTP or ddGTP), 15% (v/v) ³²P-labeled TTP (800 Ci/mmol, 10 μCi/μl), and 10% (v/v) telomerase fraction. After 1 hour at 30°C, the reactions were stopped, digested with proteinase K, ethanol precipitated, and separated as in (8). Fractions were derived from the following strains: Fig. 8, A and B: T268 [Myc⁺Δ203-101 his2-801 his2-Δ1 his3-Δ203] (77p1 SUP115; Fig. 6C, lanes 2 to 4); T268 [Fig. 8C, lanes 6 to 8: T268 TLC1-1^{His}Wt]; Fig. 6D, lanes 1 and 2, and Fig. 6E, lane 1: AVL78 [Myc⁺Δ203-101 ura3-52 pro prc pep4-1; Fig. 6D, lanes 3 and 4, and Fig. 6E, lane 2: AVL78 est2-Δ1:URA3; Fig. 6D, lanes 5 and 6, and Fig. 6E, lane 3: AVL78 tlc1-Δ1:LEU2; Fig. 6E, lanes 7, 8, and 9: T268; Fig. 6E, lanes 5 and 8: T268 est2-Δ530A; and Fig. 6E, lanes 6 and 9: T268 est2-Δ671A. The est2-Δ1 and tlc1-Δ1 deletion strains were constructed by one-step gene disruption, whereas the est2-Δ530A, est2-Δ671A, and TLC1-1^{His}Wt mutant alleles were constructed by replacement of the EST2-wt and TLC1-wt wild-type alleles, respectively. In each case, extracts were prepared for analysis after as few generations of growth as possible.
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 51. Twenty single colonies from five independent transformations of an est2-Δ strain were assayed for each mutation. The results, including the slight reduction in colony size observed for est2_{Δ530A} and est2_{Δ671A} were highly reproducible. In addition, the same phenotype of these two mutants (as well as that of the more severely affected est2_{Δ530A}, est2_{Δ671A}, and est2_{Δ671A} mutants) was enhanced in an est2_{Δ530A} strain background (38). The absence of RAD52 gene function has been shown to eliminate a backup pathway for telomere maintenance, thereby enhancing the phenotype of est mutant strains (34, 28).
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EXHIBIT 6

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Related Articles, Links

Functional analysis of HIV-1 reverse transcriptase motif C: site-directed mutagenesis and metal cation interaction.

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Motif C, present in all polymerases, has been proposed to be part of the catalytic and metal binding site of the enzyme, suggesting that polymerases have a common origin. Previously, we have shown that the metal ion manganese induces alterations in nucleotide substrate specificity in some polymerases. However, it is not known if the active site responsible for incorporation of nonspecific substrates is the same as that which incorporates specific ones. Here we show that manganese enables HIV-1 reverse transcriptase (RT) to incorporate rNTP's using RNA as a template, thus behaving as an RNA replicase. Also, we show that the mutation D186H in motif C strongly affects the natural DNA polymerase activity and that the RNA replicase activity becomes undetectable, suggesting that both activities depend on the same active site. This mutation changes the metal ion preference, with mutant RT presenting only 0.5% of the wild-type DNA polymerase activity in the presence of magnesium but 1.6% of the same activity in the presence of manganese. This variation in cation preference suggests that residue D186 is part of the metal binding site. Since residue D186 of motif C is essential for both activities and appears to be involved in the binding of an important cation needed for the specific activity, our results support the idea of a common origin for all polymerases, from an ancestral unspecified polymerase containing at least motif C.

EXHIBIT 7

Rapid Identification of All Known Retroviral Reverse Transcriptase Sequences with a Novel Versatile Detection Assay

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ABSTRACT

We have developed a highly sensitive, universal assay that allows detection as well as identification of all known retroviral reverse transcriptase (RT)-related nucleic acids in a biological sample by a single two-step experiment. The assay combines polymerase chain reaction (PCR) and reverse dot-blot hybridization (RDBH), using an array of immobilized synthetic retrovirus-specific oligonucleotides and two sets of mixed oligo primers (MOPs). These primers were derived from highly conserved motifs found in all known reverse transcriptase genes. The PCR/RDBH assay was used for qualitative analyses of human endogenous retrovirus (HERV) transcription in peripheral blood mononuclear cells (PBMCs) and in particles released by the human mammary carcinoma-derived cell line T47D. Sensitivity was further demonstrated by detection of down to 10 copies of pig endogenous retrovirus (PERV) DNA in human cDNA samples. Therefore, this assay is particularly useful for the identification of retroviral sequences in xenografts as well as in recipients of xenografted tissues and organs. Moreover, it is a valuable tool to detect retroviral transcripts and particles in cell cultures used for production of therapeutic polypeptides. The assay is further suitable for monitoring vector preparation used in human gene therapy to exclude transfer of copackaged endogenous retroviruses into target cells.

INTRODUCTION

THE GENOMES OF ALL VERTEBRATES contain a wide spectrum of endogenous retroviruses (ERVs) and reverse transcriptase (RT)-related sequences. For example, human ERVs (HERVs) are estimated to comprise at least 1–2% of the human genome.^{1,2} Although most of these sequences are assumed to be defective, some retain certain biological activities and thus represent a reservoir of retroviral genes with pathogenic potential. Characterization of particles released by the human breast cancer-derived cell line T47D revealed that complementation between several expressed HERVs can lead to pseudotype particles packaging retroviral RNA of different origin.^{3,4} Thus, activation and expression of (H)ERV may result in undesired mobilization of genetic material of retroviral ori-

gin and may interfere with the safe production of therapeutic polypeptides, with safe human gene therapy and xenotransplantation.⁵

Cross-packaging of ERVs to a high level is observed in murine packaging cells commonly used for retroviral vector preparation.⁶ Cross-packaged ERV transcripts may be transmitted to recipient cells leading to unwanted integration events, or may recombine with the vector forming new infectious retroviruses. This is of high concern for the safety of retrovirus-mediated human gene therapy. The risk of acquiring animal ERVs through xenotransplantation also requires attention since xenografts from baboons and pigs are currently discussed for human use. *In vitro* experiments indicate that xenotropic ERVs such as murine or cat retroviruses can propagate considerably in human cells.⁷ Baboon endogenous retrovirus (BaEV) read-

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ily infects human cells in culture. The same holds true for porcine ERV and several human cell types.^{3,8-10} Although these viruses do not show a pathogenic effect in their natural hosts, the situation may change when they are transferred to immunosuppressed humans, in whom the virus might replicate to high titers.^{11,12}

With respect to prospective practical applications we have established a universal detection assay that allows rapid testing of biological samples for undesired mobilization of retroviral sequences. With this method all known reverse transcriptase-related sequences of human and animal origin can be simultaneously identified in a single two-step experiment.

MATERIALS AND METHODS

RNA preparation

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) of healthy blood donors according to a guanidium isothiocyanate-caesium chloride (GIT/CsCl) ultracentrifugation protocol¹³ and dissolved in diethylpyrocarbonate (DEPC)-treated distilled water. Consecutively, mRNA was purified with Dynabeads paramagnetic particles as described by

the manufacturer (DynaL, Hamburg, Germany). Nucleic acid concentrations were calculated by spectrophotometry at 260 nm. To check for genomic DNA contaminations 50 ng of each mRNA preparation was tested in a polymerase chain reaction (PCR) with mixed oligonucleotide (oligo) primers (MOPs) omitting the reverse transcription step. Only preparations negative for DNA traces were used for PCR. Samples positive for DNA contamination were treated with RNase-free DNase (100 units/ μ g; Roche Diagnostics, Mannheim, Germany) in 100 mM sodium acetate (pH 5.0), 5 mM $MgSO_4$ until control PCR was negative.

Primers and reverse dot-blot oligonucleotides

For PCR two different mixed oligonucleotide primer (MOP) sets, MOP-1 and MOP-2, have been designed. The primer sequences correspond to highly conserved regions present in the reverse transcriptase (RT) genes of all known human endogenous and exogenous retroviruses, as well as related animal retroviruses (Fig. 1).¹⁴⁻¹⁶ MOP-1 primers preferentially amplify human and mammalian type A, B, and D reverse transcriptase sequences, whereas MOP-2 primers allow the amplification of human and mammalian type C-related RT sequences as well as RT sequences of human exogenous retroviruses such as HIV,

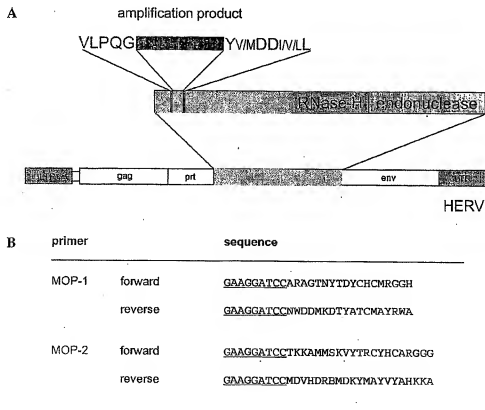


FIG. 1. (A) Localization of conserved amino acid domains in the amino-terminal coding region of reverse transcriptases of retroviruses. The core homology regions VLPQG and YM/V D DI/V/LL were used to design the mixed oligonucleotide primer sets MOP-1 and MOP-2. (B) Primer set MOP-1 was optimized for amplification of type A, B, and D retroviruses, whereas primer set MOP-2 was selected for favored priming of retrovirus type C-related templates as well as human exogenous retroviruses such as HIV, HTLV, HRV5, and foamy retroviruses. Standard single-letter abbreviations (IUPAC) are used to describe degenerate nucleotides. Both forward and reverse primers are oriented in the 5'-to-3' direction with respect to the DNA strand to be amplified.

HTLV, HRV5, and foamy viruses (Fig. 1). For each primer set a separate PCR was performed. The amplification products were then mixed in equimolar amounts and used as probe in the reverse dot-blot hybridization.

To design the oligonucleotides bound on the reverse dot-blot filters, databases were screened for RT-related sequences. RT sequences of exogenous and endogenous retroviruses were classified according to the current nomenclature and further sub-grouped with respect to their degree of nucleotide homology (data not shown). Representative members of all retrovirus families published so far were selected (Table 1) and the sequence information corresponding to the 90-bp stretch between the highly conserved RT motifs LPOG and YM/VDDI/VLL¹⁶ was used for synthesis of a pair of oligonucleotides, each 45 nucleotides in length. Thus, each dot consists of an equimolar mixture of two 45-mer oligonucleotides covering the internal sequence of the MOP amplicon.

Reverse transcription and MOP PCR

Five hundred nanograms of DNA-free mRNA preparations was reverse transcribed in a volume of 50 μ l containing 20 mM Tris-HCl (pH 8.4), 10 mM dithiothreitol (DTT), 50 mM KCl, 2.5 mM MgCl₂, deoxynucleoside triphosphates (dNTPs; 0.5 mM each), 10 units of RNasin (Promega, Madison, WI), 30 pmol of random hexamer oligonucleotides (Promega), and 20 units of murine leukemia virus (MuLV) reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 37°C for 1 hr. Consecutively, reverse-transcribed samples were denatured for 5 min at 95°C and stored at -20°C.

MOP amplification was carried out in a total volume of 50 μ l containing 1/20 of the cDNA reactions, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.001% gelatin, 50 pmol of each mixed oligonucleotide primer set, a 0.25 mM concentration of each deoxynucleoside triphosphate, and 1.25 units of *Taq* polymerase (GIBCO-BRL). Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA). Cycle parameters were as follows: 30 cycles of 94°C for 30 sec, 50°C for 4 min, and 72°C for 1 min, followed by a final extension step of 7 min at 72°C. We have chosen 50°C for the annealing step since it corresponds to the annealing temperature of the degenerate primers with the highest A-T ratio. A control reaction in which the template was omitted was carried out to detect product carryover and any traces of contaminating genomic DNA in the solutions used. Amplification products were analyzed on preparative 2.5% Tris-borate-EDTA (TBE) agarose gels and stained with ethidium bromide. Bands of interest with sizes between 100 and 150 bp corresponding to amplified retroviral reverse transcriptase sequences were excised from the gel and purified with a Gene Clean II kit (Bio 101, Vista CA). For reverse dot-blot hybridization about 50 ng of the purified fragments was labeled with [γ -³²P]dATP (3000 Ci/mmol), using a Megaprime DNA labeling kit (Amersham Pharmacia Biotech, Little Chalfont, England).

Preparation of filter arrays

Retrovirus-specific synthetic oligonucleotides corresponding to the 90-bp internal part of the amplified RT sequence were synthesized and high-performance liquid chromatography (HPLC) purified by Birnser & Grob-Biotech GmbH (Freiburg,

Germany). For each retroviral sequence 100 pmol of a pair of 45-mer oligonucleotides mixed in equimolar amounts was diluted in 5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and spotted onto ZETAProbe GT blotting membranes (Bio-Rad, Hercules, CA), using a Minifold 1 dot blotter SRC96D (Schleicher & Schuell, Dassel, Germany). Filters were rinsed in 2 \times SSC and oligonucleotides were irreversibly immobilized by UV cross-linking (Stratallinker; Stratagene, La Jolla CA). Filters were allowed to air dry.

Hybridization procedures

Standardized hybridization conditions were as follows: Pre-hybridization of reverse dot-blot filters was performed within sealed plastic bags in 0.25 M Na₂HPO₄ (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 50°C for at least 3 hr. For hybridization 5 \times 10⁵ cpm of labeled probe per milliliter of hybridization volume was added to the same solution and incubated for 16 hr under the same conditions. The membranes were then washed twice (30 min each) at 50°C in 40 mM Na₂HPO₄ (pH 7.2), 5% SDS, 1 mM EDTA and twice in 40 mM Na₂HPO₄ (pH 7.2), 1% SDS, 1 mM EDTA, respectively. Filter membranes were exposed to X-ray film (BioMax; Eastman Kodak, Rochester, NY).

RESULTS

Design of mixed oligo primers

The *pol* genes of all retroviruses and most retroelements share highly conserved core homology regions.¹⁵⁻¹⁷ Two of the most conserved amino acid regions are the VLPQG and YVM DDI/VLL motifs (Fig. 1). The spacing between both motifs is about 90 base pairs and this region shows considerably less homology when compared between different retrovirus families. According to a general principle outlined by Shih *et al.*¹⁵ we derived from these motifs universal PCR primers that allow amplification of all known retroviral RT-related templates. After comparison of RT core homology regions of all human and mammalian endogenous and exogenous retroviral sequences available in the database, two sets of degenerate *pol* primers were designed. Primer set MOP-1 was optimized for amplification of type A, B, and D retroviruses, whereas primer set MOP-2 was selected for favored priming of retrovirus type C-related templates as well as human exogenous retrovirus such as HIV, HTLV, HRV5 and foamy retroviruses. A 9-base extension featuring a clamp and a *Bam*HI restriction site was incorporated at the 5' end of each primer. Since the sequence extension has a stabilizing effect on primer-template binding kinetics, the products generated after the first PCR cycle are amplified more efficiently in the remaining cycles. Therefore, the amplification reaction can be considered as "multiplex" PCR under moderate primer-template annealing conditions. Retroviral templates in the reaction mixture can be amplified sufficiently with MOP-1 and MOP-2 primers even when the exactly matching primer is not available. Moreover, rapid product cloning for sequence verification or characterization of novel RT-related sequences is possible. PCR conditions were optimized with respect to the amount of primers, annealing time, and annealing temperature (data not shown).

TABLE 1. CLASSIFICATION OF RETROVIRUS-SPECIFIC OLIGONUCLEOTIDES AND DOT CODES

<i>Retrovirus family</i>	<i>Member</i>	<i>Sequence</i>	<i>Dot code</i>
Type B retroviruses	HERV-K(HML-1)	HML-1 (U35102)	1A
		Seq29 (S77579)	1B
	HERV-K(HML-2)	HERV-K10 (M14123)	2A
		HERV clone M3.5 (U87592)	2B
	HERV-K(HML-3)	HML-3 (U35236)	3A
		HERV1 (S66676)	3B
		RT244 (S77583)	3C
		Seq26 ^a	3D
		Seq34 ^a	4A
	HERV-K(HML-4)	Seq42 ^a	4B
		Seq43 ^a	4C
		HERV-K-T47D (AF020092)	5A
		Seq05 ^a	5B
		Seq10 ^a	5C
	HERV-K(HML-5)	HML-5 (U35161)	6A
	HERV-K(HML-6)	HML-6 (U60269)	7A
		Seq38 ^a	7B
	HERV-K(C4)	Seq56 ^a	7C
		HERV-K-C4 (U07856)	8A
	Unassigned	Seq31 ^a	8B
		SeqU39937 (U39937)	5F
Type C retroviruses	HERV-H	SeqG46.2 (AF026252)	2I, 2K ^b
		Seq61 ^a	2K, 5J ^b
	ERV9/HERV-W	Seq66 ^a	2L, 5K ^b
		ERV9 (X57147)	4E
		Seq49 ^a	4F
		Seq59 ^a	4G
		Seq60 ^a	4H
	ERV-FRD	Seq63 ^a	4I
		Seq64 ^a	4J
		HERV-W (AF009668)	4L
		ERV-FRD (U27240)	5E
		Seq46 ^a	5F
	HERV-ERI	HERV-E(4-1) (M10976)	2H
		Seq32 ^a	2I
	HERV-IP	HERV-I (M92067)	3H
		HERV-IP-T47D (U27241)	3I
	HERV-T	Seq65 ^a	3J
		S71 pCRTK1 (U12970)	2E
	MPMV	S71 pCRTK6 (U12969)	2F
		Seq36 ^a	5H
Type D retroviruses Foamy virus related	HERV-L (G895836)	HERV-L (G895836)	1E
		Seq39 ^a	1F
		Seq40 ^a	1G
		Seq45 ^a	1H
		Seq48 ^a	1I
	Unassigned human retroviral elements	Seq51 ^a	1J
		Seq58 ^a	1K
		Seq35 ^c	5G
	Human nonviral retroposon	Seq41 ^a	5I
		Seq77 ^a	5J
Human exogenous retroviruses	LINE-1 (M80343)	LINE-1 (M80343)	3L
	HRV5 (U46939)	HRV5 (U46939)	6E
	Foamy virus (Y07725)	Foamy virus (Y07725)	6F
	HTLV-I ^c	HTLV-I ^c	6G
	HTLV-II (M10060)	HTLV-II (M10060)	6H
	HIV-1 ^c	HIV-1 ^c	6I
	HIV-2 (J04542)	HIV-2 (J04542)	6J
	MMTV (M15122)	MMTV (M15122)	7E
	PERV (AF038600)	PERV (AF038600)	7F
	BaEV (D10032)	BaEV (D10032)	7G
Mammalian endogenous retroviruses	GalV (M26927)	GalV (M26927)	7H
	Mo-MuLV (J02255)	Mo-MuLV (J02255)	7I
	MPMV (ML2349)	MPMV (ML2349)	7J

^aFrom Ref. 21.^bFilter code corresponding to Fig. 4 only.^cFrom Ref. 22.

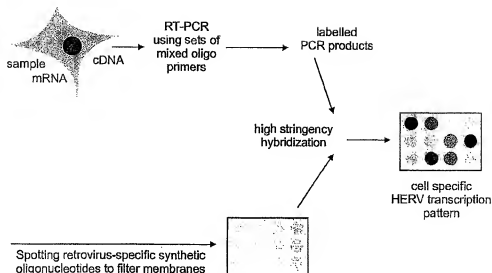


FIG. 2. Flow chart of RT-PCR/RDBH procedure described in Materials and Methods. In general, samples from any biological source, e.g., all type of body fluids, tissues, cells, and cell culture supernatants, can be tested for the presence of retroviral nucleic acids.

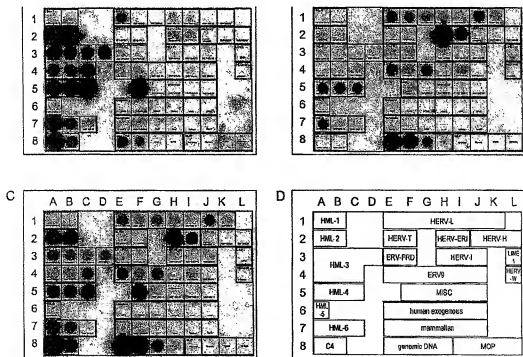


FIG. 3. HERV expression pattern in human PBMCs of a healthy blood donor. Reverse dot blots were probed under standardized conditions (as described in Materials and Methods) and DNA fragments amplified from PBMC-derived cDNA with primer set MOP-1 (A), MOP-2 (B), and with an equimolar amount of amplification products obtained with MOP-1 and MOP-2 primers, respectively (C). For fast assignment of retrovirus-specific oligonucleotides compare with (D); for origin and exact identification see Table 1.

Reverse dot-blot hybridization

In the second step, identification of the amplified products was performed by reverse dot-blot hybridization (RDBH) analysis (Fig. 2). This method is employed to sort unerringly all products of the PCR amplification. In contrast to the relaxed primer-template binding allowed during PCR amplification, RDBH enables the strict discrimination of PCR products and makes preceding false amplification of non-RT-related sequences irrelevant. The high stringency of RDBH was achieved by the use of synthetic HERV-specific oligonucleotides spotted onto the filter membranes (Table 1). These oligonucleotides correspond to the *pol* sequences amplified with MOP-1 and MOP-2 primer sets, except that they lack the primer sequences themselves. Therefore, specificity of hybridization is due solely to the amplified sequence found between the described RT core homology motifs. Thus, under high-stringency conditions the exact identification of even closely related retroviral sequences is possible.

In this study we selected 61 retrovirus-specific oligonucleotides for RDBH analysis corresponding to *pol* genes of representative members of all known human exogenous and endogenous retrovirus families. In addition, six mammalian retroviruses were included. Origin and taxonomic classification of all sequences used are summarized in Table 1.

HERV transcription pattern in human peripheral blood mononuclear cells

To assess the feasibility and specificity of the PCR/RDBH assay system, HERV transcription was analyzed in human PBMCs (Fig. 3). When the MOP-1 primer set alone was used for amplification, almost exclusively type B-related HERVs were detected, particularly members of the HERV-K subgroups HML-2, -3, -4, -6, and -C4 (Fig. 3A, dots 2A and 2B, dots 3A-3D and 4A-4C, dots 5A-5C, dots 7A and 7B, and dots 8A and 8B) and a not yet classified HERV-K-related sequence (dot 5F). This expression pattern concurs with previously published studies demonstrating a differential expression of HERV-K elements in human tissues.^{18,19} No crosshybridization was observed with type C-related HERVs. Low amounts of products were obtained for one of the human foamy virus-related HERV-L elements (Fig. 3A, dot 1E).

With MOP-2 primers HERV-E-related elements (Fig. 3B; dots 2H and 2I), sequences of the HERV-L family (dots 1E-1K), and ERV9-related HERVs (dots 4E-4G, and 4I) were preferentially amplified. A certain amount of HERV-K(HML-4)- and HERV-K(HML-6)-related sequences was also present in the hybridization probe. Although the same amount of radioactively labeled probes has been used in all hybridization reactions, genomic control DNA (dots 8E-8H) gives much stronger signals with the MOP-2-amplified probe than with the MOP-1 probe, indicating that the human genome contains significantly more copies of type C-related than type B-related HERV elements.

For detection of all retroviral sequences in a single experiment MOP-1 and MOP-2 primers were first added in an equimolar ratio to the PCR. However, this experiment resulted in a predominant amplification of type C-related sequences, the ABD-type sequences being underrepresented (data not shown). Therefore, we performed separate PCR with either MOP-1 or

MOP-2 primer sets, and mixed the purified amplification products of both reactions in equimolar amounts. This procedure resulted in a signal pattern that would have been expected when combining both primer sets (Fig. 3C) and corresponds roughly to the amount of type B- and type C-related HERV transcripts

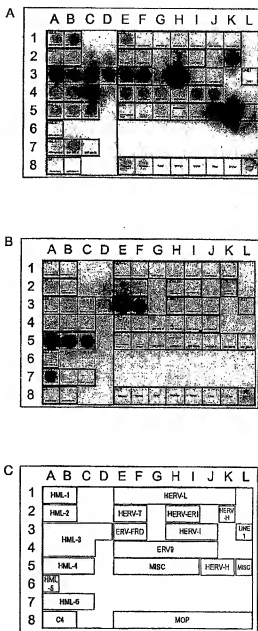


FIG. 4. HERV expression pattern in T47D cells after steroid treatment (A) and HERV transcripts packaged in T47D particles (B). DNA probes for reverse dot-blot hybridization were generated by reverse transcription of mRNA isolated from steroid-treated T47D cells (A) and from T47D particles (B), respectively.³ For fast assignment of retrovirus-specific oligonucleotides compare with (C), for origin and exact identification see Table 1.

determined previously in PBMCs by Northern analysis.²⁰ It is, however, important to note that the PCR/RDBH assay is a qualitative test, and cannot be quantified because of the use of highly degenerate primers and cross-hybridization of closely related sequences.

Detection and identification of pol sequences in retroviral particles

To evaluate the PCR/RDBH assay with respect to further practical applications, e.g., detection of small amounts of retroviral particles in preparations of therapeutic proteins, or co-packaging of endogenous retroviral sequences in vector isolates for human gene therapy, the PCR/RDBH assay was used for analysis of retroviral particles produced by the human mammary carcinoma cell line T47D. We have shown previously that particles released from this cell line after induction with steroids are pseudotypes and may package retroviral RNAs of different origins.^{3,4} T47D mRNA from steroid-treated cells (Fig. 4A) as well as particle preparations from corresponding cell culture supernatants (Fig. 4B) were therefore subjected to the PCR/RDBH analysis. Comparison of HERV transcripts expressed in T47D cells with HERV RNAs contained in T47D particles revealed that, despite a high number of transcriptionally active HERV elements in steroid-treated T47D cells, only three retroviral RNAs are packaged in the pseudotype particles. Transcripts of the HERV-K-subgroups HML-4 (Fig. 4B, dots 5A-C) and HML-6 (Fig. 4B, dot 7A) as well as type C-related ERV-FRD elements (Fig. 4B, dots 3E and 3F) were found to have accumulated preferentially in T47D particle preparations, whereas the retroviral mRNA of T47D cells contains in addition HERV-K(HML-1) (Fig. 4A, dot 1B), HERV-K(HML-3) (Fig. 4A, dots 3A-3D and 4A-4C), HERV-I (Fig. 4A, dot 3H), and HERV-H transcripts (Fig. 4A, dots 2K, 5J, and 5K) as main components. These results demonstrate the high sensitivity of the PCR/RDBH assay, since T47D cells produce only low amounts of particles not detectable by conventional methods,⁹ and suggest a high practical value for monitoring vector preparations to be used in human gene therapy.

Sensitivity and species specificity

To test further the sensitivity of the PCR/RDBH assay with respect to prospective practical use, e.g., monitoring potential transmission of porcine endogenous retroviruses (PERVs) via xenotransplantation, we performed spiking experiments with human PBMC-derived cDNA that contained serial dilutions of a cloned DNA fragment from the PERV-A *pol* region.¹⁰ Under standardized test conditions as few as 10 copies of PERV DNA were detectable in cDNA derived from 25 ng of human PBMC mRNA (Fig. 5A, dot 7F). No cross-hybridization of human-specific amplification products with the PERV-specific oligonucleotides on the filter was observed (Fig. 3C, dot 7F). Vice versa, when the PCR/RDBH test was performed with MOP-amplified pig genomic DNA as hybridization probe, no cross-hybridization of pig PCR products with human endogenous or exogenous retroviral sequences was detected (Fig. 5B). These results demonstrate the high species specificity of the PCR/RDBH assay, ruling out the possibility of pig-human retroviral interspecies cross-hybridization.

Interestingly, porcine genomic DNA seems to contain as yet unidentified PERVs that show a higher homology to murine leukemia viruses than PERVs A, B, and C, since a distinct signal was found with Moloney MuLV (Mo-MuLV) (Fig. 5B, dot 7I). A weak cross-hybridization with human genomic DNA (Fig. 5B, dots 8E and 8F) indicates that the human genome may also contain some PERV-related sequences that have not yet been characterized and therefore are not represented on the HERV dot blot.

DISCUSSION

We have established a universal PCR/RDBH detection assay that allows detection as well as identification of all known retroviral RT-related sequences in a sample in a single experiment. The assay combines a PCR using two sets of highly degenerate primers and hybridization employing an array of immobilized synthetic retrovirus-specific oligonucleotides. For

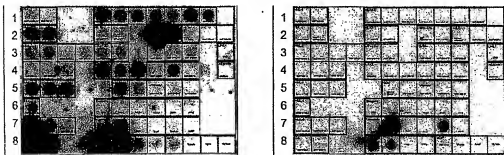


FIG. 5. HERV expression in human PBMCs after mixing human cDNA with a cloned DNA fragment containing PERV RT. Fewer than 10 copies of pig endogenous retrovirus type A DNA^{5,10} can be detected and identified under the standardized test conditions (A, dot 7F). No cross-hybridization of porcine amplification products generated from a pig DNA template can be observed with HERVs under the applied stringency conditions (B). For fast assignment of retrovirus-specific oligonucleotides compare with (D) of Fig. 3; for origin and exact identification see Table I.

primer design two of the most highly conserved motifs within the reverse transcriptase-encoding region of the *pol* gene were exploited. The usefulness of these conserved motifs for detection of novel retroviruses has been demonstrated in pioneering work by several authors.^{13,16,21,22} In the first step of our experimental approach, which can be considered as "multiplex" PCR under moderate primer-template annealing conditions, all retroviral templates of the sample investigated are amplified. In the second step, exact sorting of the amplified products is performed by RDBH under high-stringency conditions. With this highly sensitive and species-specific diagnostic tool we could detect as few as 10 PERV DNA copies contained in cDNA derived from 25 ng of human PBMC mRNA.

However, it is important to emphasize that the PCR/RDBH assay is primarily a qualitative detection technique. Although the distinct intensity of the autoradiograph signals may give a strong impression of a quantitative monitoring of retrovirus expression, it is worth noting that several parameters of uncertainty may lead to a signal pattern that differs from the true expression rates in the sample. The use of highly degenerate primer sets combined with relaxed primer-template binding conditions may lead to preferential amplification of certain "high copy" or a few "best fit" templates, whereas others stay underrepresented. This effect increases with the number of PCR cycles performed and becomes critical above 35 cycles. Thus, no more than 30 rounds of PCR should be performed. The multiplex PCR does not allow an internal standardization except for overall hybridization efficiency and autoradiograph exposure time. With PCR/RDBH identified RT related transcripts must be quantified in further experiments by conventional methods such as Northern blotting or by a specific competitive PCR established for the retroviral sequence of interest.

On the other hand, it is an advantageous feature of the PCR/RDBH assay that, because of the highly degenerate primers and cross-hybridization by lowering the stringency of hybridization conditions, it allows isolation and characterization of yet unknown retroviral sequences. DNA hybridizing to the covalently bound oligonucleotides can be eluted from the filter membrane by alkaline denaturation and reamplified to provide sufficient double-stranded DNA for cloning and subsequent sequence analysis.

With the employment of nonradioactive labeling techniques the PCR/RDBH assay offers the possibility of an automatable procedure for rapid analysis of retroviral expression. DNA chip technology may be applied, facilitating handling and increasing efficacy of reverse dot-blot filter membranes. Computer-assisted evaluation of RDBH results by phosphor/fluorescence-imaging systems may further improve visualization. It is one of the advantageous features of the PCR/RDBH assay system that the test is unlimited with respect to number and origin of retroviral RT sequences to be tested. Novel RT-encoding sequences can be easily added to the filter arrays. Modifications in the experimental design are not necessary. Moreover, by modifying the hybridization conditions PCR/RDBH can be used to search for new exo- or endogenous retroviruses with only weak homologies to already known families.

In summary, the PCR/RDBH assay is a powerful technique for precise qualitative analysis of retrovirus activity in biological samples. Number and types of retroviral sequences to be identified are determined solely by number and types of synthetic oligonu-

cleotides spotted onto reverse dot-blot membranes. PCR/RDBH could be useful in guarding patients against undesired transmission of genetic material by retroviruses from therapeutic protein preparations, in gene therapy and xenotransplantation.

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